Asialoerythropoietin, a Nonerythropoietic Derivative of Erythropoietin, Displays Broad Anti-Heart Failure Activity

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Background—We investigated the effects of asialoerythropoietin (asialoEPO), a nonerythropoietic erythropoietin derivative, on 3 murine models of heart failure with different etiologies.

Methods and Results—Doxorubicin (15 mg/kg) induced heart failure within 2 weeks (toxic cardiomyopathy). Treatment with asialoEPO (6.9 μg/kg) for 2 weeks thereafter attenuated the associated left ventricular dysfunction and dilatation. In addition, the asialoEPO-treated heart showed less myocardial fibrosis, inflammation, and oxidative damage, and diminished atrophic cardiomyocyte degeneration, which was accompanied by restored expression of GATA-4 and sarcomeric proteins. Mice with large 6-week-old myocardial infarctions exhibited marked left ventricular dysfunction with adverse remodeling (ischemic cardiomyopathy). AsialoEPO treatment for 4 weeks significantly mitigated progression of the dysfunction and remodeling and reduced myocardial fibrosis, inflammation, and oxidative damage. Finally, 25-week-old δ-sarcoglycan-deficient mice (genetic cardiomyopathy) were treated with asialoEPO for 5 weeks. AsialoEPO mitigated the progressive cardiac remodeling and dysfunction through cardiomyocyte hypertrophy, and upregulated expression of GATA-4 and sarcomeric proteins. AsialoEPO appears to act by altering the activity of the downstream erythropoietin receptor signals extracellular signal-regulated protein kinase, Akt, signal transducer, and activator of transcription 3 and 5 in a model-specific manner.

Conclusions—The findings suggest that asialoEPO exerts broad cardioprotective effects through distinct mechanisms depending on the model, which are independent of the erythropoietic action. This compound may be promising for the treatment of heart failure of various etiologies. (Circ Heart Fail. 2012;5:274-285.)

Key Words: cardiomyopathy ■ erythropoietin ■ heart failure

Erythropoietin (EPO) is a hypoxia-induced hormone that is essential for normal erythropoiesis and is used widely in patients with anemia. Notably, however, the EPO receptor is also expressed on cells within the cardiovascular system, including cardiomyocytes and endothelial cells, suggesting EPO exerts cardiovascular effects beyond hematopoiesis.1-3 For example, recombinant human EPO exerts cardioprotective effects in hearts subjected to acute myocardial infarction or ischemia-reperfusion injury, that is, EPO administration prior to or during myocardial ischemia significantly enhances functional recovery after reperfusion.4,5 In addition, EPO administered during the chronic stage of myocardial infarction acts via a different mechanism to mitigate the cardiac dysfunction and remodeling caused by the old myocardial infarction.6 EPO also appears to exert a protective effect against heart disease of nonischemic origin, for example doxorubicin cardiomyopathy.7 Taken together, 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. That said, hemoglobin levels do increase with EPO administration, which might offset its beneficial effects to some degree due to the related increases of blood viscosity and pressure.8

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Asialoerythropoietin (asialoEPO) was developed by removing the sialic acid moieties from EPO.9 Because the function of the sialic acid is to delay the clearance of EPO in

Received November 24, 2011; accepted February 10, 2011.
From the Department of Cardiology (T.T., G.T., H.K., T.K., A.O., T.W., K.M., A.T., K.G., R.M., H.U., M.K., S.M.) and Department of Orthopedics (K.Y.), Gifu University Graduate School of Medicine, Gifu; Life Science Research Center, Gifu University, Gifu (H.N.); Department of Food Science, Kyoto Women’s University, Kyoto (T.F.); Department of Cardiology, Hyogo Prefectural Amagasaki Hospital, Hyogo, Japan (H.F.).

The online-only Data Supplement is available with this article at http://circheartfailure.ahajournals.org/lookup/suppl/doi:10.1161/CIRCHEARTFAILURE.111.965061/-/DC1.

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Circ Heart Fail is available at http://circheartfailure.ahajournals.org

DOI: 10.1161/CIRCHEARTFAILURE.111.965061

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vivo, this modification gives asialoEPO a very short half-life, one that is insufficient to significantly stimulate hematopoiesis. On the other hand, only brief exposure to EPO is needed for its tissue-protective effects. In a study performed by Erbayraktar et al, for example, asialoEPO exhibited neuroprotective effects in experimental models of brain and spine injury, without an effect on hemoglobin levels. This concept was further validated in other tissues, including kidneys.

Administration of asialoEPO also protected against ischemia reperfusion injury in the heart, possibly through mechanisms involving inhibition of apoptosis. In addition, we recently demonstrated that asialoEPO attenuates nephrectomy induced left ventricular remodeling and dysfunction without increasing hemoglobin levels. These findings underscore the fact that the EPO-mediated improvement of cardiac function is not mediated through increased hemoglobin levels, but through pleiotropic effects.

We therefore hypothesized that systemically administered asialoEPO would exert therapeutic effects on models of established heart failure having different etiologies by acting through cardioprotective mechanisms that do not involve increasing hemoglobin levels. To test that idea, we examined the effect of asialoEPO on doxorubicin induced toxic cardiomyopathy, postcardiac infarction ischemic cardiomyopathy, and δ-sarcoglycan-deficient genetic cardiomyopathy. We also investigated the mechanisms specifically involved in each type of heart failure.

Methods

See the online-only Data Supplement for additional details.

AsialoEPO

AsialoEPO was prepared from EPO as previously described. The 6.9 μg asialoEPO was synthesized from 1500 IU EPO; they are equimolar. EPO at the dose of 1500 IU/kg is known to be within the therapeutic range as previously reported. Thus, we applied 6.9 μg/kg asialoEPO to the mice at every administration.

Animal Models and Experimental Protocols

This study conforms to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH publication No. 85–23, revised 1996), and was approved by the Institutional Animal Research Committee of Gifu University. We prepared 3 murine models of heart failure with different etiologies (1) toxic cardiomyopathy induced by doxorubicin, (2) ischemic cardiomyopathy due to a large old myocardial infarction, and (3) genetic cardiomyopathy with δ-sarcoglycan gene deficiency.

Statistical Analysis

Values were shown as means±SEM. The significance of the differences in variance was evaluated using Bartlet test. When the variance was significantly different, the significance of differences was tested by Kruskal-Wallis test. A Wilcoxon signed rank test was used for the comparison of hemoglobin levels. Otherwise, it was evaluated using 1-way ANOVA with a posthoc Newman-Keul multiple comparison test, or a repeated-measures ANOVA when analyses were within group, including the time factor. The statistical analyses on hemodynamic data indeed included all groups, but the data of EPO-treated groups were omitted from Figures 1–6 of the printed manuscript to avoid complexity (but separately shown in the online Supplemental Figure; see online-only Data Supplement). Values of P<0.05 were considered significant.

Results

EPO Receptor in the Heart

The mouse heart showed the EPO receptor gene at the intermediate level between fibroblasts and kidney. Western blot and immunohistochemistry demonstrated EPO receptor on cardiomyocytes and vascular cells in the heart (online-only Data Supplement Figure 1).

Effect of AsialoEPO on Toxic Cardiomyopathy

A single intraperitoneal injection of doxorubicin (15 mg/kg) induced an established heart failure in mice within 2 weeks (Figure 1A); asialoEPO at a dose of 6.9 μg/kg was administered twice a week for the subsequent 2 weeks. Hemoglobin levels were unaffected by the 2-week asialoEPO treatment protocol: 14.7±0.3 g/dL (median, 14.8; range, 13.0–16.7) before treatment versus 14.5±0.3 g/dL (median, 14.6; range 12.8–15.6) after treatment, P=0.59. On the other hand, the doxorubicin induced left ventricular dysfunction and dilatation were significantly attenuated in the asialoEPO-treated group, as compared with control (Figures 1A and 1B). Histologically, treatment with doxorubicin caused atrophic degeneration of the cardiomyocytes, myocardial fibrosis, an inflammatory reaction with leukocyte infiltration, and oxidative damage (Figure 1C). The sarcomeric proteins myosin heavy chain and troponin I, as well as their transcription factor GATA-4, were downregulated (Figure 2A), which may be the molecular mechanism underlying the doxorubicin induced atrophic degeneration of cardiomyocytes. AsialoEPO significantly restored the expression of those proteins as well as cardiomyocyte size (Figures 1C and 2A). In addition, it attenuated the myocardial fibrosis and leukocyte infiltration seen in hearts treated with doxorubicin (Figure 1C). By contrast, cardiac levels of transforming growth factor-β1, a fibrogenic cytokine, and tumor necrosis factor-α, an inflammatory cytokine, were not affected by either doxorubicin or asialoEPO (Figures 2B and 2C).

Doxorubicin induced oxidative damage also was attenuated by asialoEPO, as indicated by reductions of both 8-hydroxy-2'-deoxyguanosine and 4-hydroxyl-2-nonenal (Figures 1C and 2B). Myocardial capillary density was not affected by either doxorubicin or asialoEPO, nor was myocardial expression of vascular endothelial growth factor (VEGF; Figures 1C and 2B). Electron microscopy revealed significant degeneration of cardiomyocytes from doxorubicin-treated mice. This included myofibrillar disorganization and loss and accumulation of mitochondria showing deformities, swelling, or degeneration (mitochondriosis; Figure 1D). All of these features were attenuated significantly by asialoEPO.

The prevalence of in situ nick end-labeling (TUNEL)-positive nuclei in cardiomyocytes and noncardiomyocytes was similar in hearts from sham-injected and doxorubicin-injected mice, and was not affected by treatment with asialoEPO (Figure 2D). Consistent with that finding, activation of cardiac caspase-3 was not observed in any group (Figure 2E), and we did not detect ultrastructure indicative of apoptosis among cardiomyocytes in any group.

Effect of AsialoEPO on Ischemic Cardiomyopathy

Large myocardial infarctions were induced in mice by ligating the left coronary artery. Treatment with asialoEPO (6.9 μg/kg,
twice a week) was started 6 weeks postinfarction, by which time marked left ventricular dysfunction with severe dilatation was apparent (Figure 3A), and the treatment was continued for 4 weeks. This treatment protocol had no effect on hemoglobin levels: 14.3 ± 0.4 g/dL (median, 14.3; range, 12.3–16.3) before treatment versus 14.7 ± 0.4 g/dL (median, 14.8; range, 13.3–16.5) after treatment, *P* = 0.99. Postinfarction cardiac remodeling and dysfunction worsened over time in the saline-treated controls, but were significantly mitigated in the asialoEPO-treated group (Figure 3). The beneficial effects of asialoEPO on macroscopic structure and function were supported on a microscopic scale by reductions in myocardial fibrosis, inflammation, and oxidative damage (Figure 3C), which were accompanied by reductions in myocardial expression of transforming growth factor-β1, tumor necrosis factor-α, and an oxidative marker 4-hydroxyl-2-nonenal (Figures 4B and C). On the other hand,

![Figure 1. Physiological and histopathologic findings in doxorubicin induced cardiotoxicity. A, Echocardiographic findings. LVEd indicates left ventricular diameter at diastole; LVDS, left ventricular diameter at systole; %FS, percentage of fractional shortening. B, Cardiac catheterization data. LVSP indicates left ventricular peak systolic pressure; LVEDP, left ventricular end diastolic pressure; +dP/dt, maximum and minimum first derivative of left ventricular pressure. C, Photographs of the histological and immunohistochemical preparations and graphs showing quantitative morphometry. Bars, 1 mm in the uppermost panels and 20 μm in the others. D, Electron micrographs of cardiomyocytes obtained from doxorubicin-injected mice without (upper panel) and with asialoEPO treatment (lower panel). Asterisk in the upper panel indicates degenerated myofibrils. Bars, 1 μm. *P* < 0.05 versus the sham-injected group without asialoEPO treatment; # *P* < 0.05 versus the doxorubicin-injected group without asialoEPO treatment.](http://circheartfailure.ahajournals.org/cover)
cardiomyocytes were markedly hypertrophied in the infarcted heart, especially at the border of the infarct area, and asialoEPO showed no effect. Myocardial expression of myosin heavy chain, troponin I, and GATA-4 also was not affected (Figures 3C and 4A). AsialoEPO significantly increased both capillary vessel density at the border zone (Figure 3C) and myocardial VEGF expression in infarcted hearts (Figure 4B). Electron microscopy revealed the degenerative findings, that is, myofibrillar loss and mitochondriosis, were attenuated in cardiomyocytes bordering the infarct area in asialoEPO-treated mice (Figure 3D). The prevalence of TUNEL-positive nuclei among cardiomyocytes or noncardiomyocytes was similar in hearts with and without infarction, and was not affected by treatment with asialoEPO (Figure 4D). Myocardial activation of caspase-3 was not observed (Figure 4E), and we detected no apoptotic cardiomyocytes under electron microscopy in any group.

**Effect of AsialoEPO on Genetic Cardiomyopathy**

The left ventricular cavity was more dilated and its function was more reduced in 25-week-old δ-sarcoglycan-deficient...
mice than in age-matched wild-type mice (Figure 5A). Beginning at 25 weeks, asialoEPO (6.9 μg/kg) was administered twice a week for the subsequent 5 weeks. Again, the treatment protocol had no effect on hemoglobin levels in the cardiomyopathic mice: 14.1 ± 0.4 g/dL (median, 14.1; range, 12.1–16.1) before treatment versus 14.3 ± 0.4 g/dL (median, 14.3; range, 12.3–16.3) after treatment, P = 0.57, but it significantly mitigated the progressive cardiac remodeling and
dysfunction (Figures 5A and 5B). The heart-to-body weight ratio, which was increased in \( \alpha \)-sarcoglycan-deficient mice, returned to normal during the treatment with asialoEPO. Cardiomyocyte size was increased in the mutant mice and further increased by asialoEPO (Figure 5C). Myocardial fibrosis, which is increased in the hearts of the \( \alpha \)-sarcoglycan-deficient mice, was attenuated by treatment with asialoEPO (Figure 5C). Interestingly, myocardial expression of GATA-4, myosin heavy chain, and troponin I was downregulated in the \( \alpha \)-sarcoglycan-deficient mice, but was restored by asialoEPO (Figure 6A). Myocardial inflammation and oxidative damage were apparent in the mutant mice, and were not affected by asialoEPO (Figures 5C, 6B, and 6C). Treatment with asialoEPO also had no effect on capillary density or myocardial VEGF expression, which did not differ between the wild-type and mutant mice (Figures 5C and 6B). Most cardiomyocytes from \( \alpha \)-sarcoglycan-deficient mice showed severe degeneration, including loss of myofibrils and mitochondria, and some presented vacuolar degeneration with autophagic vacuoles under an electron microscope (Figure 5D). Treatment with asialoEPO attenuated those features. The prevalence of TUNEL-positive nuclei among cardiomyocytes was also reduced by asialoEPO (Figure 5D).

**Figure 4.** Molecular biological findings in the postinfarction heart. A, Western blots of myosin heavy chain and troponin I and their transcription factor, GATA-4. B, Western blots of transforming growth factor-\( \beta \)-1, 4-hydroxyl-2-nonenal, and vascular endothelial growth factor. C, ELISA for tumor necrosis factor-\( \alpha \). D, In situ nick end-labeling assays separately evaluated in myocytes and nonmyocytes. E, Western blots of caspase-3. F, Western blots of the downstream mediators of EPO receptor signaling: extracellular signal-regulated protein kinase, Akt, signal transducer and activator of transcription (STAT)3, and STAT5. *\( P < 0.05 \) versus the sham-operated group without asialoEPO treatment; #, \( P < 0.05 \) versus the postinfarction group without asialoEPO treatment.
Figure 5. Physiological and histopathologic findings in δ-sarcoglycan-deficient cardiomyopathy. A, Echocardiographic findings. LVEDd and LVEDs indicate left ventricular diameter at diastole and systole; %FS, percentage of fractional shortening. B, Cardiac catheterization data. LVSP and LVEDP indicate left ventricular peak systolic and end-diastolic pressure; ±dP/dt, maximum and minimum first derivative of left ventricular pressure. C, Photographs of the histological and immunohistochemical preparations and graphs showing the quantitative morphometry. Bars, 1 mm in the uppermost panels and 20 μm in the others. D, Electron micrographs of cardiomyocytes obtained from a wild-type mouse without asialoEPO treatment (upper panel) and δ-sarcoglycan-deficient mice without (middle panel) and with asialoEPO treatment (lower panel). Bars, 1 μm. *P<0.05 versus the wild-type without asialoEPO treatment; #P<0.05 versus the δ-sarcoglycan-deficient group without asialoEPO treatment.
cytes or noncardiomyocytes was similar in the wild-type and \( \delta \)-sarcoglycan-deficient mouse, and was not affected by treatment with asialoEPO (Figure 6D). No activation of myocardial caspase-3 was observed (Figure 6E), and no apoptotic ultrastructure was detected in cardiomyocytes in any group.

### Difference in Effects Between EPO and AsialoEPO on Heart Failure Models

The efficacy of asialoEPO on cardiac function, geometry, and histology was similar to that of the equimolar EPO in each heart failure model (online-only Data Supplement Figures II–IV). Distinct from EPO, however, asialoEPO showed no erythrogenesis.

### Downstream Signals of the Erythropoietin Receptor

Phosphatidylinositol 3-kinase/Akt, receptor-associated Janus family tyrosine kinase/signal transducer and activator of transcription (STAT), and extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK) are all known to be downstream mediators of erythropoietin.
receptor signaling in cardiac cells both in vitro and in vivo. Among the 3 models tested, hearts with doxorubicin cardiomyopathy showed less ERK activity than sham-injected hearts. By contrast, postinfarction hearts showed greater ERK and Akt activity than sham-operated hearts (Figures 2F and 4F), which is consistent with earlier studies.6,7 And whereas hearts from δ-sarcoglycan-deficient mice showed less ERK activity than hearts from wild-type mice, they showed higher levels of Akt activity (Figure 6F). Treatment with asialoEPO significantly restored ERK activity in doxorubicin cardiomyopathy without affecting Akt, STAT3, or STAT5 activity (Figure 2F). In hearts with old infarctions, where ERK and Akt were activated, treatment with asialoEPO further activated both and also activated STAT3 and STAT5 (Figure 4F). Finally, asialoEPO significantly restored ERK activity in δ-sarcoglycan cardiomyopathy and further increased Akt activity, but did not affect the activities of STAT3 or STAT5 (Figure 6F).

**Discussion**

The results of the present study show the beneficial effects of asialoEPO on 3 models of heart failure with different etiologies: doxorubicin cardiotoxicity (toxic origin), postinfarction heart failure (ischemic origin), and δ-sarcoglycan-deficient cardiomyopathy (genetic origin). Importantly, asialoEPO showed no erythropoiesis that is possibly detrimental, despite the efficacy on the heart being similar to EPO. In addition, we previously reported that asialoEPO also has beneficial effects in renal dysfunction (chronic kidney disease [CKD])-associated heart failure model.14 Collectively, these findings suggest asialoEPO exerts broad cardioprotective effects that could make it a useful tool for the treatment of heart failures with various etiologies. In addition, our data indicate the presence of EPO receptor in the mouse heart, inconsistent with the recent study by Sinclair et al16 but consistent with the former ones.17,18 Our studies thus confirm the notion that the cardioprotective effects of asialoEPO are mediated via signaling downstream of the EPO receptor and are independent of its erythropoietic action.

**Mechanisms Underlying the Beneficial Effects of AsialoEPO**

The 3 models of heart failure studied exhibit phenotypes that are very distinct from one another (Table 1). Doxorubicin cardiotoxicity is characterized by atrophic degeneration of cardiomyocytes, inflammation, oxidative damage, and fibrosis, but not by altered vessel density. The postinfarction heart is characterized by cardiomyocyte hypertrophy with degeneration, inflammation, oxidative damage and fibrosis, and reduced vessel density. Delta-sarcoglycan-deficient cardiomyopathy is characterized by cardiomyocyte hypertrophy with degeneration, increased fibrosis, little or no inflammation or oxidative damage, and no change in vessel density. AsialoEPO appears to work on each type of heart failure through multiple actions summarized in Table 1. These actions include (1) mitigation of cardiomyocyte degeneration, (2) reduction of inflammation and oxidative damage, (3) reduction of fibrosis, and (4) increased angiogenesis. These various effects are likely all related to one another.

Cardiomyocyte degeneration was observed in all 3 heart failure models studied. Although apoptosis among cardiomyocytes may be an aggravating factor in heart failure, our TUNEL assays and electron microscopy indicate that cardiomyocyte apoptosis is not important in any of the present models. Instead, affected cardiomyocytes showed severe degenerative changes, including myofibrillar derangement, disruption, and loss, as well as proliferation of subcellular organelles (mitochondriosis). All of these changes were mitigated by asialoEPO, which also restored GATA-4 expression in 2 of the models. Because GATA-4 is a key transcription factor regulating expression of cardiac sarcomeric proteins (eg, myosin heavy chain and troponin I), it seems plausible that its downregulation underlies the observed sarcomeric disintegration. We previously reported that asialoEPO restored GATA-4 expression in CKD-associated heart failure.14 We suggest that the beneficial effects of asialoEPO on GATA-4 expression, myofibrillar content, and sarcomeric integrity are key elements underlying the observed improvement in cardiac function in the failing hearts, as these changes would directly influence the contractile power of individual cardiomyocytes.

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**Table 1. Phenotypes Observed in Three Types of Heart Failure and the Effect of AsialoEPO on These Phenotypes**

<table>
<thead>
<tr>
<th></th>
<th>Doxorubicin Cardiotoxicity</th>
<th>Postinfarction Heart</th>
<th>δ-Sarcoglycan Cardiomyopathy</th>
<th>Nephrectomy-Induced Heart Failure*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype vs Sham on Phenotype</td>
<td>Effect of AsialoEPO on Phenotype</td>
<td>Effect of AsialoEPO on Phenotype</td>
<td>Effect of AsialoEPO on Phenotype</td>
<td>Effect of AsialoEPO on Phenotype</td>
</tr>
<tr>
<td><strong>Cardiomyocyte:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>↓ Restoration</td>
<td>↑ No effect</td>
<td>↑ Further increase</td>
<td>↑ Further increase</td>
</tr>
<tr>
<td>Degeneration</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
</tr>
<tr>
<td>Inflammation and oxidative damage</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
<td>↑ No effect</td>
<td>↑ Attenuation</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
<td>↑ Attenuation</td>
</tr>
<tr>
<td>Vasculature</td>
<td>→ No effect</td>
<td>↓ Restoration</td>
<td>→ No effect</td>
<td>↓ Restoration</td>
</tr>
</tbody>
</table>

*Data on nephrectomy-induced heart failure from Ogino et al.14

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*Phenotype vs Wild, † Phenotype vs Sham, *Data on nephrectomy-induced heart failure from Ogino et al.14

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Induction of powerful inflammatory mediators is reportedly associated with heart failure.\textsuperscript{19,20} Inflammatory reactions often are associated with oxidative stress in a vicious cycle in which inflammatory mediators and various oxidants induce and exacerbate one another. Inflammation usually is followed by reactive fibrosis that can lead to ventricular diastolic dysfunction.\textsuperscript{21} The present heart failure models all exhibit some or all of the features of active myocardial inflammation, oxidative damage, and fibrosis, and all were significantly attenuated by asialoEPO. These results confirm the notion that, like EPO, asialoEPO displays pleiotropic effects, including anti-inflammatory, antioxidative, and antifibrotic effects.

Reduced myocardial capillary density has been shown to play a significant role in cardiac remodeling in some types of heart failure, including those caused by pressure overload and end stage renal disease.\textsuperscript{22,23} The angiogenic activity of EPO already has been reported in postinfarction heart failure.\textsuperscript{24} In addition, 1 recent study reported that induction of VEGF via the STAT3 pathway is a molecular mechanism underlying EPO-induced angiogenesis.\textsuperscript{25} Moreover, we confirmed that both EPO and asialoEPO restore expression of VEGF and increase STAT3 activation and angiogenesis in CKD-associated heart failure.\textsuperscript{14} Another recent study reported that EPO induces angiogenesis to protect the postinfarction heart through sonic hedgehog signaling.\textsuperscript{26} Both angiogenesis and upregulated VEGF expression were noted in the present postinfarction model, which is compatible with that earlier study,\textsuperscript{24} and both were further increased by asialoEPO. On the other hand, we observed that neither was affected in the sham or wild-type; the former could have improved systolic function and the latter mainly diastolic function of the heart.

Heart Failure Model-Specific Activation of Downstream Mediators of EPO Receptor Signaling and their Regulation by AsialoEPO

Phosphatidylinoositol 3-kinase/Akt, Janus family tyrosine kinase/STAT, and ERK/MAPK are all known to be downstream mediators of EPO receptor signaling in cardiac cells.\textsuperscript{4} It is noteworthy that both mediator type and the degree of activation differed greatly among the 3 heart failure models tested (Table 2). We found that ERK is primarily inactivated in δ-sarcoglycan-deficient cardiomyopathy and in doxorubicin cardiotoxicity, which is consistent with earlier studies,\textsuperscript{27,28} whereas it is activated in postinfarction hearts and in CKD-associated heart failure.\textsuperscript{14} We also found that asialoEPO restored or further augmented ERK activity in all models, which suggests ERK activation plays an important role in the failing myocardium. Consistent with that idea, activated ERK phosphorylates GATA-4 to enhance its DNA binding and transcriptional activation.\textsuperscript{29} Similarly, a study using isolated rat heart subjected to excessive left ventricle wall stress (induced by balloon inflation) showed MAPK (p38 and ERKs) to be involved in activation of GATA-4 binding to DNA.\textsuperscript{30} In addition, activated ERK negatively regulates the ubiquitin-proteasome system and autophagy, thereby inhibiting protein degradation,\textsuperscript{31,32} and we recently showed that ubiquitination of sarcomeric proteins is augmented in doxorubicin cardiotoxicity, but is attenuated on restoration of ERK activity.\textsuperscript{33} In summary, diminished ERK activity appears to be important for stimulating GATA-4-dependent sarcomeric protein synthesis and inhibiting ubiquitin-dependent sarcomeric protein degradation.

Downstream mediators of EPO receptor signaling were differentially affected by treatment with asialoEPO in the 3 heart failure models (Table 2). Although it is difficult to directly correlate mediator activity with the affected phenotypes, we previously showed that the anti-inflammatory and antioxidant effects of EPO were respectively mediated via STAT and Akt activation.\textsuperscript{7} In addition, the angiogenic activity of EPO is associated, at least in part, with induction of VEGF, which is mediated via a STAT pathway.\textsuperscript{25} Further studies to elucidate the molecular signals and the corresponding phenotypes in heart failure and their regulation by asialoEPO would seem warranted.

### Table 2. Activation Status of the Downstream Mediators of EPO Receptor Signaling in Three Types of Heart Failure and the Effect of AsialoEPO on Their Activity

<table>
<thead>
<tr>
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</thead>
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<tr>
<td></td>
<td>Activity vs Sham</td>
<td>Effect of AsialoEPO on the Activity</td>
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<td>Effect of AsialoEPO on the Activity</td>
</tr>
<tr>
<td>ERK</td>
<td>↓</td>
<td>Restoration</td>
<td>↑</td>
<td>Further increase</td>
</tr>
<tr>
<td>Akt</td>
<td>→</td>
<td>No effect</td>
<td>↑</td>
<td>Further increase</td>
</tr>
<tr>
<td>STAT3</td>
<td>→</td>
<td>No effect</td>
<td>→</td>
<td>Increase</td>
</tr>
<tr>
<td>STAT5</td>
<td>→</td>
<td>No effect</td>
<td>→</td>
<td>Increase</td>
</tr>
</tbody>
</table>

AsialoEPO indicates asialoerythropoietin; ERK, extracellular signal-regulated protein kinase; STAT, signal transducer and activator of transcription; ↓, less activity than in the sham or wild type; ↑, greater activity than in the sham or wild type; →, the activity is similar to the sham or wild type.

*Data on the nephrectomy-induced heart failure from Ogino et al.\textsuperscript{14}
Although we believe our findings are fairly comprehensive, it is unfortunate that none of these descriptions establish any cause and effect relationships with respect to the mechanisms of asialoEPO. A dose escalating study with the compound may bring about more convincing results. Another effective means of resolving the issue may be through the use of conditional knockout or transgenic mice expressing an EPO receptor gene or its downstream signal genes. Further investigation is warranted.

Clinical Implications
Recent clinical studies have raised serious questions about the beneficial effect of EPO induced improvement of anemia on the incidence of death and cardiovascular or renal events.14–36 EPO induces erythropoiesis, which does indeed reduce systemic hypoxia. However, it also causes polycythemia, increases platelet aggregability, and may activate the renin-angiotensin and endothelin systems, resulting in hypertension, though it does appear to have direct protective effects on the cardiovascular system. Given its multiple effects, it is difficult to sort out the underlying mechanisms that explain the clinical outcomes of patients treated with EPO. Our previous and present studies have shown that asialoEPO exerts a protective effect against various types of heart failure through mechanisms unrelated to relief of anemia. This is reminiscent of the beneficial effects of asialoEPO in various neurological disorders, including focal ischemia of brain, spinal cord compression, and sciotic nerve compression.11 Our findings thus imply that a short-lived, nonerythrogenic EPO derivative could be a useful therapeutic agent for the treatment of patients with heart failure of various etiologies.

Conclusions
We have shown here that asialoEPO exerts a protective effect against various types of heart failure with different etiologies in mice, that is, toxic, ischemic, and genetic cardiomyopathies, through mechanisms unrelated to relief of anemia. A direct antidegenerative effect on cardiomyocytes and antiﬁbrosis on myocardium appeared to be underlying mechanisms, according to the affected phenotypes commonly seen in each heart failure model.

Acknowledgments
We thank Drs Yoshiyuki Moriguchi and Hideki Saito (Chugai Pharmaceutical Co., Ltd.) for providing us with technical advice, and the staff of Kyoto Women’s University (Yumi Azuma, Erina Ogawa, Chika Kuzumoto, Yoriko Kubo, Yuka Saito, and Sawako Takeuchi) for technical assistance.

Sources of Funding
Supported in part by the grants from Gifu University.

Disclosures
None.

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Clinical Perspective

Recent clinical studies have raised serious questions about the beneficial effect of erythropoietin (EPO) induced improvement of anemia on the incidence of death and cardiovascular or renal events. EPO induces erythropoiesis, which does indeed reduce systemic hypoxia. However, it also causes polycythemia, increases platelet aggregability, and may activate the rennin-angiotensin and endothelin systems, resulting in hypertension, though it does appear to have some direct protective effects on the cardiovascular system. Given its multiple effects, it is difficult to parse the underlying mechanisms that explain the clinical outcomes of patients treated with EPO. Asialoerythropoietin (asialoEPO) is an EPO derivative, but is too short-lived in the plasma to result in erythropoiesis. We have shown here that asialoEPO exerts a protective effect against various types of heart failure with different etiologies in mice, that is, toxic, ischemic, and genetic cardiomyopathies, through mechanisms unrelated to relief of anemia. A direct antidegenerative effect on cardiomyocytes and antifibrosis on myocardium appeared to be contributing mechanisms according to the affected phenotypes commonly seen in each heart failure model. AsialoEPO altered the activity of the downstream EPO receptor signals extracellular signal-regulated protein kinase, Akt, and signal transducer and activator of transcription 3 and 5 in a model-specific manner. The findings suggest that asialoEPO exerts broad cardioprotective effects through distinct mechanisms depending on the model, which are independent of erythropoietic action. This compound may be promising for the treatment of heart failure of various etiologies.
Asialoerythropoietin, a Nonerythropoietic Derivative of Erythropoietin, Displays Broad Anti-Heart Failure Activity


*Circ Heart Fail*. 2012;5:274-285; originally published online February 16, 2012; doi: 10.1161/CIRCHEARTFAILURE.111.965061

*Circulation: Heart Failure* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1941-3289. Online ISSN: 1941-3297

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SUPPLEMENTAL MATERIAL

Asialoerythropoietin, A Nonerythropoietic Derivative of Erythropoietin, Displays Broad Anti-Heart Failure Activity

Supplemental Methods

AsialoEPO. AsialoEPO was prepared from EPO as previously described. The 6.9 µg asialoEPO was synthesized from 1500 IU EPO; they are equimolar. EPO at the dose of 1500 IU/kg is known to be within the therapeutic range as previously reported. Thus, we applied 6.9 µg/kg asialoEPO to the mice at every administration.

Animal Models and Experimental Protocols. This study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Institutional Animal Research Committee of Gifu University.

1) Toxic Cardiomyopathy Model. Cardiomyopathy was induced in 10-week-old male C57BL/6J mice (CLEA Japan) by a single intraperitoneal injection of doxorubicin (doxorubicin hydrochloride; Kyowa Hakko) at a dose of 15 mg/kg. We previously confirmed that, within 2 weeks at that dose, doxorubicin induces cardiomyopathy both functionally and histologically in all mice not receiving a therapeutic intervention. After an echocardiographic examination, the mice were randomly divided into a saline-treated (control) group (n=12), EPO-treated group (1500 IU/kg, subcutaneous injection twice a week for 2 weeks, n=6) and asialoEPO-treated group (6.9 µg/kg, subcutaneous injection twice a week for 2 weeks, n=10). The assignment resulted in
identical echocardiographic data between groups (Figure 1A and Supplementary Figure IIB). In sham-injected mice, a volume of saline equivalent to the doxorubicin volume was intraperitoneally injected, and 2 weeks later the mice were similarly treated with saline or asialoEPO (n=6 each).

2) Ischemic Cardiomyopathy Model. Male C57BL/6J mice (8-10 weeks of age, CLEA Japan) were initially anesthetized with 2% halothane in a mixture of N₂O and O₂ (0.5 L/min each) via a nasal mask, after which they intubated with a 20G intravenous catheter and ventilated with 0.5% halothane in a mixture of N₂O (0.1 L/min) and O₂ (0.5 L/min) using a rodent ventilator. Myocardial infarction was induced by occlusion of the left coronary artery as described previously. Six weeks after the operation, 27 mice remained alive, and they received an echocardiographic examination and were randomly divided into a saline-treated (control) group (n=10), EPO-treated group (1500 IU/kg, subcutaneous injection twice a week for 2 weeks, n=6) and an asialoEPO-treated group (6.9 µg/kg, subcutaneous injection twice a week for 4 weeks, n=11). The assignment was found to result in identical echocardiographic data between groups (Figure 3A and Supplementary Figure IIIB). Sham-operated animals underwent the same surgical procedures except the left coronary artery was not occluded. All of the sham-operated mice were alive 6 weeks later and were administered saline or asialoEPO (n=6) in a similar manner.

3) Genetic Cardiomyopathy Model. δ-Sarcoglycan-deficient mice (B6.129-Sgcdtm1Mcm/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and bred as previously described. δ-Sarcoglycan-deficient mice develop cardiomyopathy as well as progressive muscular dystrophy, which is seen morphologically as extensive areas of necrosis/fibrosis in the heart. Notably, one family and two sporadic cases of human dilated cardiomyopathy were recently identified in which the patients presented with mutations in the δ-sarcoglycan gene. Administration of saline (n=8), EPO (1500 IU/kg, subcutaneous injection twice a week
for 2 weeks, n=6) or asialoEPO (6.9 µg/kg, subcutaneous injection twice a week for 5 weeks, n=8) was started when the male animals reached 25 weeks of age. The assignment to treatments were performed in a random manner, which was found to result in identical echocardiographic values between groups (Figure 5A and Supplementary Figure IVB). Nontransgenic littermates (C57BL/6J, CLEA Japan) were used as controls and administered saline or asialoEPO (n=6 each) in a similar manner.

**Hemoglobin Measurement.** Hemoglobin was evaluated using HemoCue Hb 201+ (HemoCue) with blood from the tail vein before and after treatment.

**Physiology Studies.** Physiological studies (echocardiography and cardiac catheterization) were carried out under anesthesia with halothane (induction, 2%; maintenance, 0.5%) in a mixture of N₂O and O₂ (0.5 L/min each) via a nasal mask as described previously. Echocardiography was performed just before administration of asialoEPO and before sacrifice, while cardiac catheterization was done only before sacrifice because of its invasiveness.

**Histological Analysis.** Once the physiological measurements were complete, mice were killed by cervical dislocation 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 (Aldrich). 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 (x400) in each section using a multipurpose color image processor (LUZEX F, Nireco).
Immunohistochemistry. Four-µm-thick deparaffinized sections or 8-µm-thick cryosections were incubated with an antibody against panleukocyte antigen (CD45, Pharmingen), 8-hydroxy-2'-deoxyguanosine (8-OHdG; a commonly used marker of oxidative damage to DNA, Japan Institute of the Control of Aging), Flk-1 (Santa Cruz) or EPO receptor (Santa Cruz, M-20). An ABC kit (Dako) was used for immunostaining with DAB as the chromogen, and nuclei were counterstained with hematoxylin. The primary antibodies were substituted with the respective control IgG in the control sections.

To evaluate the incidence of apoptosis among cardiomyocytes, we labeled with rhodamine-phalloidin in combination with in situ nick end-labeling (TUNEL) using Fluorescein-FragEL (Oncogene). Immunofluorescently stained preparations were observed under a confocal microscope (LSM510, Zeiss).

Electron Microscopy. Cardiac ultrastructure was examined under a transmission electron microscope (H-800; Hitachi) using conventional methods as previously described.

Western Blotting. Lysates from heart tissues (n=3-5 from each group) were used for Western blot analysis. Proteins were separated and transferred to membranes using standard protocols, after which they were probed using antibodies against myosin heavy chain, troponin I, their transcriptional factor GATA-4, troponin I, transcriptional factor GATA-4, transforming growth factor-β1 (TGF-β1; Promega), 4-hydroxyl-2-nonenal (4-HNE; a marker of oxidative damage to cell membrane, NOF corporation), vascular endothelial growth factor (VEGF; Santa Cruz), caspase-3 (Cell Signaling) and EPO receptor (Santa Cruz, M-20). Activation of extracellular signal-regulated protein kinase (ERK), Akt, signal transducer and activator of transcription 3 (STAT3) and STAT5, four downstream mediators of EPO receptor signaling, was assessed using antibodies against...
their phosphorylated forms: p-ERK, p-Akt, p-STAT3 and p-STAT5 (all from Cell Signaling). The blots were visualized using chemiluminescence (ECL; Amersham), and the signals were quantified by densitometry. α-tubulin (analyzed using an antibody from Santa Cruz) served as the loading control.

**Enzyme-Linked Immunosorbent Assays (ELISAs).** Myocardial levels of tumor necrosis factor-α (TNF-α) were assayed using an ELISA (R&D Systems). Three to five hearts from each group were used for this assay.

**RNA Preparation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR).** Total RNA was isolated from the normal mouse heart and the control samples –i.e., normal mouse kidneys and NIH3T3 cells (embryonic fibroblasts purchased from the American Type Culture Collection), using a RNeasy mini kit (Qiagen). Total RNA was reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems). The quantitative real-time PCR analysis of EPO receptor mRNA using the fluorescent SYBR green method (Bio-Rad) was performed in accordance with the manufacturer’s instructions; the primers used were: forward, 5’-GGGCTCCGAAGAACTTCTGTG-3’ and reverse, 5’-TGACTTTCGTGACTCACCCTC-3’ (from the GenBank DNA sequence database, the National Center for Biotechnology Information). The data generated from each reaction were subjected to a gene expression analysis using an iCycler iQ Real-Time PCR Detection System (Bio-Rad).

**Statistical Analysis.** Values were shown as means±SEM. The significance of the differences in variance was evaluated using Bartlett test. When the variance was significantly different, the significance of differences was tested by Kruskal-Wallis test. A Wilcoxon signed-rank test for the comparison of hemoglobin levels. Otherwise, it
was evaluated using one-way ANOVA with a post-hoc Newman-Keul's multiple comparison test or a repeated-measures ANOVA when analyses were within-group including the time factor. The statistical analyses on hemodynamic data indeed included all groups but the data of EPO-treated groups were omitted from figures of the printed manuscript to avoid complexity (but separately shown in the online Supplemental Figures). Values of P<0.05 were considered significant.
References


Supplemental Figures

Supplemental Figure 1

A

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Arbitrary Unit</th>
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<tr>
<td>Fibroblast</td>
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<tr>
<td>Kidney</td>
<td>1±0.15</td>
</tr>
<tr>
<td>Heart</td>
<td>0.63±0.05</td>
</tr>
</tbody>
</table>

B

Heart

EPO Receptor (84-78 kDa)

C

Anti-EPO Receptor Antibody (-)  Anti-EPO Receptor Antibody (+)

Vessel
Supplemental Figure 2

A. Hemoglobin levels (g/dl)

B. LVEDd (mm)  LVEDs (mm)  EF(%)  HR(bpm)

C. HW/BW  Mean Size (μm)  % Fibrosis  CD45+ Cells (μm²)

8-OHdG Cells (HPF)  FJc + Cells (HPF)
Supplemental Figure 3

A) Hemoglobin levels (g/dl)

B) LVEDd (mm)  
LVEDs (mm)  
EF (%)  
HR (bpm)

C) H/VEWI  
Myocardial Stain (μm)  
% Fibrosis  
CD45+ Cells  
8-OHdG+ Cells  
F4/80+ Cells
Supplemental Figure 4
Supplemental Figure Legends

Supplemental Figure 1
Detection of EPO receptor.
(A) RT-PCR.  (B) Western blot.  (C) Immunohistochemistry.  Bars, 20 µm.

Supplemental Figure 2
Physiological and histopathological findings in doxorubicin-induced cardiotoxicity.
(A) Hemoglobin levels.  (B) Echocardiographic and cardiac catheterization data.
LVDd and LVDs, left ventricular diameter at diastole and systole; %FS, %fractional shortening.
LVSP and LVEDP, left ventricular peak systolic and end-diastolic pressure; ±dP/dt, maximum and minimum first derivative of left ventricular pressure.
(C) Quantitative morphometry from the histological and immunohistochemical preparations.  #p<0.05 compared with the doxorubicin-injected group without treatment.

Supplemental Figure 3
Physiological and histopathological findings in the postinfarction heart.
(A) to (C): same as in Supplemental Figure 2.  #p<0.05 vs. the postinfarction group without treatment.

Supplemental Figure 4
Physiological and histopathological findings in δ-sarcoglycan-deficient cardiomyopathy.
(A) to (C): same as in Supplemental Figure 2.  #p<0.05 vs. the δ-sarcoglycan-deficient group without treatment.