Adaptive response of the heart to long-term anemia induced by iron deficiency

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Naito Y, Tsujino T, Matsumoto M, Sakoda T, Ohyanagi M, Masayama T. Adaptive response of the heart to long-term anemia induced by iron deficiency. Am J Physiol Heart Circ Physiol 296: H585–H593, 2009. First published January 9, 2009; doi:10.1152/ajpheart.00463.2008.—Anemia is common in patients with chronic heart failure and an independent predictor of poor prognosis. Chronic anemia leads to left ventricular (LV) hypertrophy and heart failure, but its molecular mechanisms remain largely unknown. We investigated the mechanisms, including the molecular signaling pathway, of cardiac remodeling induced by iron deficiency anemia (IDA). Weanling Sprague-Dawley rats were fed an iron-deficient diet for 20 wk to induce IDA, and the molecular mechanisms of cardiac remodeling were evaluated. The iron-deficient diet initially induced severe anemia, which resulted in LV hypertrophy and dilation with preserved systolic function associated with increased serum erythropoietin (Epo) concentration. Cardiac STAT3 phosphorylation and VEGF gene expression increased by 12 wk of IDA, causing angiogenesis in the heart. Thereafter, sustained IDA induced upregulation of cardiac hypoxia inducible factor-1α gene expression and maintained upregulation of cardiac VEGF gene expression and cardiac angiogenesis; however, sustained IDA promoted cardiac fibrosis and lung congestion, with increased serum Epo concentration and cardiac STAT3 phosphorylation after 20 wk of IDA compared with 12 wk. Upregulation of serum Epo concentration and cardiac STAT3 phosphorylation is associated with a beneficial adaptive mechanism of anemia-induced cardiac hypertrophy, and later decreased levels of these molecules may be critical for the transition from adaptive cardiac hypertrophy to cardiac dysfunction in long-term anemia. Understanding the mechanism of cardiac maladaptation to anemia may lead to a new strategy for treatment of chronic heart failure with anemia.

Iron deficiency has been reported as a common cause of anemia in patients with advanced HF (18). A clinical study has also shown that treatment with iron supplements in patients with HF is beneficial (20, 28). Thus iron deficiency anemia (IDA) is obviously important not only as a primary cause, but also as a facilitator, of HF. Numerous studies have shown that IDA leads to left ventricular (LV) hypertrophy in developing rats (16, 21, 22, 27), but no data about its molecular mechanisms, including the molecular signaling pathways, of IDA-induced cardiac remodeling, are available.

Recent studies have shown that the Epo receptor (EpoR) is expressed in a variety of cells, including myocardium (30). Moreover, Epo-EpoR signaling can stimulate the Jak/STAT, MAPK, and phosphatidylinositol-3-kinase/Akt signaling pathways in hematopoietic and cardiac cells (23). Epo treatment improves quality-of-life scores and LV ejection fraction in patients with HF (14).

Therefore, in the present study, we investigated the molecular mechanism, including the Epo-EpoR signaling pathway, of cardiac remodeling in long-term anemia induced by iron deficiency.

MATERIALS AND METHODS

Animals

Protocol 1. Male 4-wk-old Sprague-Dawley (SD) rats (n = 60) were randomly assigned to the iron-deficient and the control diet groups. Regular rat chow was supplemented with ~0.003% FeC6H5O7·H2O. The iron-deficient group (n = 30) was fed a diet that was not supplemented with FeC6H5O7·H2O (prepared by Oriental Yeast, Chiba, Japan) and drank deionized water to reduce the influence of water iron content for 20 wk; the control group (n = 30) was fed the regular rat chow and drank regular water for 20 wk. Rats were maintained on a 12:12-h light-dark cycle and had free access to food and water. After 1, 4, 12, and 20 wk of each diet, the rats were anesthetized with pentobarbital sodium, and blood samples and tissues were collected.

Protocol 2. After 12 wk on the iron-deficient diet, 12 rats were randomized to one of the following treatment groups: 6 animals were treated with the Epo analog darbepoetin-α (Kirin Pharma, Tokyo, Japan; 1.5 μg·kg−1·wk−1), as previously described (24), and 6 animals received vehicle (PBS). Darbepoetin-α and vehicle were injected intraperitoneally once per week. After 8 wk, the rats were evaluated by echocardiography, and blood and tissue samples were collected. All experimental procedures were approved by the Animal Research Committee of Hyogo College of Medicine.

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Assessments of Blood Pressure, Hemoglobin, Serum Concentrations of Iron, Epo, and TNF-α, and Renal Function

Systolic blood pressure (SBP) and heart rate were measured with a noninvasive computerized tail-cuff system (MK-2000, Muromachi Kikai, Tokyo, Japan). Blood hemoglobin was measured by the sodium lauryl sulfate-hemoglobin method. Serum concentrations of iron, blood urea nitrogen, and creatinine were determined by the 2-nitroso-5-[N-n-propyl-N-(3-sulfopropyl)amino]phenol method, the urease-glutamate dehydrogenase-UV method, and enzymatic assay (creatinine amidohydrolase-sarcosine oxidase-peroxidase method), respectively. Serum Epo concentration was determined by radioimmunoassay as previously described (11). Serum concentration of TNF-α was measured with the Bio-Plex suspension array system (Bio-Rad, Hercules, CA). Twenty-four-hour urine samples were collected in metabolic cages for measurement of urinary volume, protein, and electrolyte levels.

Echocardiography

Rats were anesthetized with ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg) and evaluated by transthoracic echocardiography. We measured LV cavity size and wall thickness and calculated LV fractional shortening as previously described (15) with a 12-MHz phased-array transducer (Aploio, Toshiba Medical Systems, Odawara, Japan). LV end-diastolic and end-systolic dimensions (LVDD and LVDs) and LV anterior and posterior wall thicknesses were measured using M-mode traces. We also recorded pulsed-Doppler mitral flow-velocity pattern and measured peak early diastolic filling velocity (E), peak filling velocity at atrial contraction (A), the E-to-A ratio, and deceleration time, as previously described (15).

RNA Extraction and Real-Time Quantitative RT-PCR

Total RNA was extracted from the LV using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruc-
Histological Analysis

Midpapillary slices from the hearts were fixed with buffered 4% paraformaldehyde, embedded in paraffin, and cut into 4-μm-thick sections. Serial sections were stained with hematoxylin-eosin, Masson’s trichrome, and Picro Sirus red. Photomicrographs were quantified with the use of NIH Image J software to measure the cross-sectional area of 100 randomly selected cardiomyocytes in the LV and assess the area of fibrosis in the myocardium. Midpapillary slices from the heart samples were immunohistochemically stained with von Willebrand factor antibody (1:1,000 dilution; Dako, Kyoto, Japan). The number of von Willebrand factor-positive vessels was counted to calculate the number of microvessels per cardiomyocyte.

Statistical Analysis

Values are means ± SE. Statistical analysis was performed using one-way ANOVA or Student’s t-test. Differences among three groups were assessed by Tukey-Kramer multiple comparison test. Differences were considered significant at $P < 0.05$.

RESULTS

Effects of IDA on Physiological Parameters

The iron-deficient diet induced anemia measured by hemoglobin content (g/dl) in all groups (Fig. 1A). There were no significant differences in body weight in any group until 4 wk; however, body weight decreased significantly in the IDA group compared with the control group after 12 wk and thereafter (404 ± 13 vs. 457 ± 12 g, $P < 0.05$; Fig. 1B), suggesting that growth was inhibited by iron deficiency. SBP was also comparable between the control group and the IDA group until 4 wk and became progressively decreased in the IDA group compared with the control group after 12 wk, while heart rate was significantly higher in the IDA group than the control group at 4 wk but became comparable in the control group and the IDA group after 12 wk (Fig. 1, C and D). Chronic IDA led to reductions of body weight and blood pressure.

Effects of IDA on Cardiac Function

At 12 and 20 wk, the heart was larger in the IDA group than the control group (Fig. 1E). A marked increase in the LV weight-to-tibia length ratio in the IDA group compared with the control group was observed at 12 wk and thereafter, demonstrating cardiac hypertrophy (Fig. 1F). Wet lung weight-to-tibia length ratio and wet-to-dry lung weight ratio, an index of pulmonary congestion (13), were significantly increased after 20 wk in the IDA group relative to the control group (Fig. 1, G and H). Sustained IDA induced cardiac hypertrophy, resulting in cardiac dysfunction.

Representative M-mode echocardiographic traces and pulsed-Doppler mitral flow-velocity patterns are shown in Fig. 2A. LV hypertrophy developed gradually and reached a peak at 12 wk in the IDA group. LV dilatation was evident at 12 wk and was more enhanced at 20 wk in the IDA group, whereas fractional shortening slightly increased in the IDA group compared with the control group after 12 wk, but the...
differences were not statistically significant (Fig. 2, B–E). The E wave was higher in the IDA group than the control group at 12 and 20 wk, whereas the E-to-A ratio was lower and deceleration time was prolonged in the IDA group relative to the control group after 12 wk. Conversely, the E wave and E-to-A ratio were increased and deceleration time was shortened in the IDA group relative to the control group after 20 wk (Fig. 2, F–H).

Histological analysis showed that the cross-sectional area of cardiomyocytes was increased in the IDA group compared with the control group at 12 wk and thereafter (Fig. 3, A and D). Marked interstitial fibrosis was not detected in the LV of the IDA group after 12 wk; however, it appeared progressively in the IDA group compared with the control group after 20 wk (Fig. 3, B, C, and E).

Serum Concentrations of Iron and Epo and Cardiac EpoR Expression in IDA-Induced Cardiac Remodeling

Serum iron concentration was significantly lower in the IDA group than in the control group even at 1 wk, whereas serum Epo concentrations progressively increased until 12 wk but decreased at 20 wk in the IDA group (Fig. 4, A and B). Cardiac EpoR gene expression was increased at 12 wk and thereafter in the IDA group (Fig. 4C).

Gene Expression and Phosphorylation of Signal Pathway in IDA-Induced Cardiac Remodeling

The iron deficient diet induced an increased expression of fetal-type cardiac genes, including those for Nppa, in the heart of the IDA group at 4 wk and thereafter (Fig. 5A). Cardiac

![Fig. 3. Histological analysis of IDA-induced cardiac remodeling. A–C: representative images of heart sections stained with hematoxylin and eosin (A), Masson's trichrome (B), and Picro Sirius red (C). Scale bars, 50 μm. D and E: quantitative analysis of cardiac myocyte cross-sectional area and myocardial interstitial fibrosis in rats fed control (open bars; n = 6) or iron-deficient (solid bars; n = 6) diet. *P < 0.05 vs. control at corresponding time point.](http://ajpheart.physiology.org/)
Nppb gene expression was upregulated in the IDA group compared with the control group at 12 wk but decreased at 20 wk in the IDA group (Fig. 5D). In contrast, phosphorylations of Akt and phosphorylation of ERK did not differ between the control group and the IDA group (Fig. 5, E and F).

Cardiac VEGF and HIF-1α Gene Expression and Angiogenesis in IDA-Induced Cardiac Remodeling

Cardiac VEGF gene expression did not differ between the control group and the IDA group until 4 wk; however, its expression was upregulated in the IDA group relative to the control group at 12 and 20 wk (Fig. 5A). Cardiac HIF-1α gene expression decreased with aging in the control group, which is in agreement with a previous study (29). HIF-1α gene expression was comparable between the control group and the IDA group until 12 wk but was upregulated at 20 wk in the hearts of the IDA group (Fig. 5B). Cardiac HIF-1α protein expression was upregulated at 20 wk in the IDA group (Fig. 5C). The number of microvessels per cardiomyocyte increased at 12 and 20 wk in the IDA group.

Renal Function and Serum TNF-α Levels in Sustained IDA

Since the cardiorenal syndrome and TNF-α play an important role in the anemia observed in patients with HF (19), we evaluated renal function and serum TNF-α levels in both groups (Table 1). Serum blood urea nitrogen levels and urinary sodium excretion were increased in the IDA group compared with the control group, while urinary potassium excretion was decreased in the IDA group at 12 and 20 wk. In addition, urinary volume and serum TNF-α levels were increased only at 20 wk in the IDA group compared with the control group.

In summary, IDA, initially induced in LV hypertrophy with preserved systolic function, increased serum Epo concentrations, cardiac STAT3 phosphorylation, and VEGF gene expression. Over time, however, the effects of IDA resulted in cardiac fibrosis and lung congestion, with decreased serum Epo levels and cardiac STAT3 phosphorylation.

Effects of Epo on IDA-Induced Cardiac Remodeling

To further explore whether the preservation of the increased levels of Epo are associated with adaptive cardiac remodeling, we finally evaluated the cardiac effect of chronic Epo therapy on the rats after 12 wk on the iron-deficient diet. The administration of Epo attenuated the downregulation of cardiac STAT3 phosphorylation (Fig. 7A) and prevented cardiac dysfunction in the IDA group (Fig. 7, B–G). Meanwhile, blood hemoglobin and SBP were not altered in the Epo-treated IDA group: 3.5 ± 0.3 and 4.4 ± 0.3 g/dl blood hemoglobin and 94 ± 6 and 91 ± 8 mmHg SBP in the Epo-treated IDA group and the untreated IDA group, respectively. The preservation of the increased levels of Epo prevents the transition from adaptive cardiac hypertrophy to cardiac dysfunction in sustained IDA.

DISCUSSION

Chronic anemia is common among patients with HF, relating to increased morbidity and mortality (2, 12). Moreover, chronic severe anemia is known to cause HF. However, the mechanisms, including the molecular signaling pathway, of HF in chronic severe anemia are not fully resolved. We tried to...
reveal the mechanisms behind the adaptive and maladaptive response of the heart to long-term anemia. The new finding of the present study is that the increased serum Epo concentration and cardiac STAT3 phosphorylation play a compensatory role in the beneficial cardiac remodeling induced by chronic IDA. In other words, a decrease in these molecules may be critical for the transition from adaptive cardiac hypertrophy to cardiac dysfunction in long-term anemia caused by iron deficiency.

Cardiac Remodeling Induced by Sustained IDA

IDA led to a reduction in SBP at 12 wk and induced cardiac hypertrophy, whereas IDA led to cardiac dysfunction at 20 wk. The presence of cardiac dysfunction at 20 wk was characterized by the increase in LV weight-to-tibia length ratio and wet-to-dry lung weight ratio, the increased Nppa, Nppb, and collagen type 3 gene expression, and the increased myocardial interstitial fibrosis. To our knowledge, this is the first report to evaluate the time course of cardiac function in IDA-induced cardiac remodeling with echocardiography. In particular, the serial changes in the pulsed-Doppler mitral flow-velocity pattern were notable. At 4 wk, cardiac hypertrophy and LV dilatation could not be found; however, both were evident at 12 wk. What is significant here is that the fractional shortening was maintained until 20 wk. The E-to-A ratio was low and the deceleration time was prolonged at 12 wk; however, the E wave and E-to-A ratio were increased with the shortened deceleration time at 20 wk. Together with our physiological results, our model of IDA-induced cardiomyopathy may be able to show diastolic HF at 20 wk.

Possible Role of Serum Epo and Cardiac STAT3 Phosphorylation in IDA-Induced Cardiac Remodeling

Anemia causes systemic vasodilatation, decreased blood viscosity, and sodium and water retention, resulting in high cardiac output (31). IDA induces LV eccentric hypertrophy in developing rats (16, 21, 22, 27); however, the molecular mechanism responsible for LV remodeling in this setting has been unknown. Recent studies showed that Epo treatment improves quality-of-life scores and LV ejection fraction in patients with HF (14), indicating that Epo has protective effects on cardiac diseases. In addition, EpoR is expressed in a variety of cells not directly involved in erythropoiesis, including myo-cardium (30). In the present study, we found a dynamic variation in serum Epo levels and cardiac EpoR expression in the process of LV hypertrophy in high cardiac output induced by IDA. A recent study reported that the Epo-EpoR system in the nonhematopoietic cells plays an important protective role against pressure-overload cardiac hypertrophy (4). Thus the cardiac Epo-EpoR system seems to protect against not only

**Fig. 5.** Increased gene expression and phosphorylation of signal pathway in IDA-induced cardiac remodeling in hearts of rats fed control (open bars; n = 6) or iron-deficient (solid bars; n = 6) diet. A–C: time course of atrial natriuretic peptide (nppa), brain natriuretic peptide (nppb), and collagen type 3 gene expression. D–F: representative Western blot (top) and densitometric (bottom) analysis of phosphorylated (p) and native STAT3, Akt, and ERK expression. Expression of nppa, nppb, and collagen type 3 genes was normalized to endogenous 18S rRNA gene expression, and relative levels of gene expression are plotted. Expression of phosphorylated STAT3, Akt, and ERK was standardized on the basis of native STAT3, Akt, and ERK expression, and relative levels of expression are plotted. *P < 0.05 vs. control at corresponding time point. †P < 0.05 vs. 12 wk after iron-deficient diet. &P < 0.05 vs. 4 wk after iron-deficient diet. $P < 0.05 vs. 1 wk after control diet. ¶P < 0.05 vs. 4 wk after control diet.
concentric hypertrophy induced by pressure overload but, also, eccentric hypertrophy induced by volume overload. IDA induced cardiac hypertrophy and led to an enhancement in the cardiac Epo-EpoR system at 12 wk. In contrast, serum Epo levels even decreased and myocardial fibrosis markedly increased at 20 wk. In view of these findings, Epo is required to maintain cardiac function in the model of IDA-induced cardiac remodeling. The mechanisms of downregulation of serum Epo levels are unknown. At 20 wk after the change in diet, mild renal insufficiency (slight but significant increase in urinary protein excretion) and increased serum TNF-α concentration were observed. Thus these factors may reduce renal Epo secretion in the IDA group (10).

Moreover, we found marked phosphorylation of STAT3, but not Akt and ERK, in IDA-induced hypertrophied heart. In addition, the variations in the increased phosphorylation of STAT3 in the heart correlated with those in the circulating Epo levels. Epo-EpoR signaling can stimulate the Jak/STAT, MAPK, and phosphatidylinositol-3-kinase/Akt signaling pathways in hematopoietic and cardiac cells (23). Although there is no direct evidence, enhanced phosphorylation of STAT3 via Epo may be involved in IDA-induced hypertrophied heart, and such signaling appeared to play a compensatory role in IDA-induced cardiac remodeling. A recent report showed an altered phosphorylation of STAT3, but not Akt and ERK, signaling in pressure-overload cardiac hypertrophy in transgene-rescued...
Epo receptor-null mutant mice (4). While serum Epo levels and phosphorylation of STAT3 peaked at 12 wk and decreased at 20 wk, cardiac EpoR expression remained to be increased until 20 wk, suggesting a compensatory mechanism for the decreased Epo-EpoR signaling in the heart of the IDA group. Epo and hypoxia have been shown to synergistically induce EpoR expression in the cultured endothelial cells (5). Additionally, TNF-α has been shown to upregulate EpoR expression (7). In the present study, cardiac HIF-1α gene expression and serum TNF-α levels further increased at 20 wk compared with 12 wk in the IDA group. Thus the hypoxia-HIF-1α system and TNF-α may have enhanced EpoR expression in the chronic stage to compensate for the declining serum Epo concentration.

Epo has an angiogenic effect (25). However, von Willebrand factor-positive cells were observed in the heart of the IDA group even after serum Epo levels decreased at 20 wk, suggesting that some signals other than Epo might have been involved in the neovascularization of IDA heart in the chronic phase. HIF-1α is known to enhance VEGF expression and, finally, contribute to neovascularization (26). Thus HIF-1α may have promoted neovascularization at 20 wk by regulating VEGF in the heart of the IDA group.

Conclusions

The upregulation of serum Epo concentration and cardiac STAT3 phosphorylation is associated with the beneficial adaptive mechanism of anemia-induced cardiac hypertrophy. As a result, it can be concluded that downregulation of these molecules is critical for the transition from adaptive cardiac hypertrophy to cardiac remodeling in long-term anemia. Therefore, further understanding of the mechanism of cardiac maladaptation to anemia should lead to a new therapeutic strategy in chronic heart failure with anemia.

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