Erythropoietin Activates Mitochondrial Biogenesis and Couples Red Cell Mass to Mitochondrial Mass in the Heart

Martha S. Carraway, Hagir B. Suliman, W. Schuyler Jones, Chien-Wen Chen, Abdelwahid Babiker, Claude A. Piantadosi

Rationale: Erythropoietin (EPO) is often administered to cardiac patients with anemia, particularly from chronic kidney disease, and stimulation of erythropoiesis may stabilize left ventricular and renal function by recruiting protective effects beyond the correction of anemia.

Objective: We examined the hypothesis that EPO receptor (EpoR) ligand-binding, which activates endothelial NO synthase (eNOS), regulates the prosurvival program of mitochondrial biogenesis in the heart.

Methods and Results: We investigated the effects of EPO on mitochondrial biogenesis over 14 days in healthy mice. Mice expressing a mitochondrial green fluorescent protein reporter construct demonstrated sharp increases in myocardial mitochondrial density after 3 days of EPO administration that peaked at 7 days and surpassed hepatic or renal effects and antedced significant increases in blood hemoglobin content. Quantitatively, in wild-type mice, complex II activity, state 3 respiration, and mtDNA copy number increased significantly; also, resting energy expenditure and natural running speed improved, with no evidence of an increase in left ventricular mass index. Mechanistically, EPO activated cardiac mitochondrial biogenesis by enhancement of nuclear respiratory factor-1, PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α), and mitochondrial transcription factor-A gene expression in wild-type but not in eNOS−/− or protein kinase B (Akt1)−/− mice. EpoR was required, because EpoR silencing in cardiomyocytes blocked EPO-mediated nuclear translocation of nuclear respiratory factor-1.

Conclusions: These findings support a new physiological and protective role for EPO, acting through its cell surface receptor and eNOS-Akt1 signal transduction, in matching cardiac mitochondrial mass to the convective O2 transport capacity as erythrocyte mass expands. (Circ Res. 2010;106:1722-1730.)

Key Words: cardiac metabolism ■ erythropoietin ■ nitric oxide ■ Akt1/protein kinase B ■ mitochondrial biogenesis
for mitochondrial turnover and proliferation (e.g., during exercise, cell stress, energy deficits, calorie restriction, and disease mitigation) and is under calcium and redox regulation.\textsuperscript{12–15} Specialized nuclear transcription factors, such as nuclear respiratory factor (NRF)-1 and NRF-2, along with PGC-1 (peroxisome proliferator-activated receptor γ coactivator 1) family coactivators are required for mitochondrial gene activation and for mtDNA replication and transcription. This program is also stimulated by Akt, which is known to be activated by EpoR as well as to activate eNOS. This background suggested the novel possibility that EPO induces mitochondrial biogenesis through EpoR-dependent activation of eNOS.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Mice**

The studies were approved by the Institutional Animal Care and Use Committee and conducted in young male C57BL/6J, Akt1\textsuperscript{−/−}, eNOS\textsuperscript{−/−}, and mitochondrial green fluorescent protein (GFP) reporter mice bred in our vivarium.\textsuperscript{16} Mice were injected subcutaneously with human recombinant EPO or an equal volume of 0.9% NaCl once daily for 3 consecutive days, and tissues were removed under general anesthesia at the indicated times.

**Microscopy**

Formalin-fixed and paraffin-embedded tissue blocks were sectioned (5 \(\mu\)mol/L), mounted on glass slides, and deparaffinized. Laser-scanning confocal microscopy was performed on a Zeiss LSM 410 microscope and fluorescence imaging on a Nikon microscope through a 520 nm filter. The fluorescence signals were quantified using computer software.

**Proteins**

Cardiac proteins were resolved by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes and probed with primary antibodies (Santa Cruz Biotechnology, Santa Cruz, Calif). After application of the primary antibody, the membranes were washed and incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology and Jackson) and developed in ECL (Santa Cruz Biotechnology), and densitometry was performed on digitized images. At least 4 samples were used at each time point. Pooled cardiac mitochondria were prepared and respiration measured at 35°C\textsuperscript{17} using calibrated Clark mini-electrodes (Diamond General, Ann Arbor, Mich).\textsuperscript{18}

**RNA and DNA**

Cytoplasmic RNA was isolated and cDNA synthesized using the Invitrogen SUPERSCRIPT System. Cardiac mtDNA was isolated using NaI kits. Mouse-specific primers and probes were designed as reported\textsuperscript{19} RNA samples (1 \(\mu\)g) were reverse-transcribed, and gene transcripts were amplified in triplicate with gene-specific primers\textsuperscript{19,20} and quantified by densitometry normalized to 18S rRNA or GAPDH. mtDNA copy number was determined as reported.\textsuperscript{21}

**Metabolic Measurements**

Steady-state O2 consumption and CO2 production (\(V_{\text{O2}}\) and \(V_{\text{CO2}}\)) were measured in resting mice at the same time of day\textsuperscript{13} in individual metabolic chambers by collecting expired gas at a timed flow rate.
Voluntary Exercise
Mice were housed individually in cages containing rodent exercise wheels equipped with a digital magnetic counter activated by wheel rotation. To limit the variability in time spent running, individual mice were observed initially for 72 hours and then assigned randomly to groups that were well-matched for running duration, running speed, and distance in kilometers were recorded daily.

Expired gas concentrations were measured by gas chromatography, 

\[ V_{\text{O}_2} \] and \[ V_{\text{CO}_2} \] were computed with standard formulae, and resting energy expenditure (REE) was calculated with a modified Weir formula and expressed in kcal/d.\(^2\)

Table 1. Blood Hemoglobin Concentration After Hypoxic Hypoxia or EPO Administration

<table>
<thead>
<tr>
<th>Time (Days)</th>
<th>Wt</th>
<th>eNOS (-)/</th>
<th>Akt1 (-)/</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.3 ± 0.2</td>
<td>14.1 ± 0.3</td>
<td>13.7 ± 0.3</td>
</tr>
<tr>
<td>7</td>
<td>17.7 ± 0.3(^<em>) (16.3 ± 0.4(^</em>))</td>
<td>16.1 ± 0.2(^<em>) (15.9 ± 0.4(^</em>))</td>
<td>...</td>
</tr>
<tr>
<td>14</td>
<td>19.7 ± 0.3(^<em>) (17.0 ± 1.4(^</em>))</td>
<td>18.8 ± 1.0(^<em>) (16.6 ± 0.7(^</em>))</td>
<td>... (15.1 ± 0.2(^*))</td>
</tr>
</tbody>
</table>

Means ± SEM hemoglobin values in g/dL (n = 4–9 Wt mice) measured after exposure to 18,000 feet (0.5 ATA) of altitude for 0, 7, or 14 days or after the 3-day EPO regimen (values in parentheses; \(^*\) \(P<0.05\) vs control; \(n\) = 4 at each point).

Tissue Distribution and Mass of Mitochondria
The distribution and mass of mitochondria in the tissues of mitochondrial-targeted GFP reporter mice were examined before and after EPO administration (Figure 1). In reporter mice before EPO, most of the tissues demonstrated fairly uniform, low-intensity, green fluorescence with rare bright cells containing abundant mitochondria. After 3 days of EPO administration, however, the heart showed intense mitochondrial fluorescence throughout the myocardium (Figure 1A an 1B), whereas the other tissues showed modest, nonuniform enhancement of mitochondrial fluorescence, eg, skeletal mus-

**Results**

**Mitochondrial Biogenesis in Reporter Mice Before and After EPO**
The distribution and mass of mitochondria in the tissues of mitochondrial-targeted GFP reporter mice were examined before and after EPO administration (Figure 1). In reporter mice before EPO, most of the tissues demonstrated fairly uniform, low-intensity, green fluorescence with rare bright cells containing abundant mitochondria. After 3 days of EPO administration, however, the heart showed intense mitochondrial fluorescence throughout the myocardium (Figure 1A an 1B), whereas the other tissues showed modest, nonuniform enhancement of mitochondrial fluorescence, eg, skeletal mus-

![Figure 2. Cardiac EpoR expression and Akt1, ERK1/2, and HO-1 activation by EPO and cardiac EpoR transcript levels by real-time RT-PCR after 3 days of EPO administration to Wt mice.](image-url)

Figure 2. Cardiac EpoR expression and Akt1, ERK1/2, and HO-1 activation by EPO and cardiac EpoR transcript levels by real-time RT-PCR after 3 days of EPO administration to Wt mice. A, EpoR mRNA was detected before EPO and increased after 3 days of EPO administration to Wt mice (P<0.05 vs control; n = 4 at each point). B, EpoR localization in mouse hearts before and after EPO treatment (red fluorescence) with DAPI nuclear counterstain (blue). C, EPO activation of Akt1 in mouse heart. Phospho-/total Akt was increased in Wt heart after 3 days of EPO administration and fell to control by day 14. Phospho-/total Akt was unresponsive to EPO in eNOS \(-\)/ mice. Negative control are heart tissue from Akt1 \(-\)/ mouse hearts. D, Phospho-/total ERK1/2 activation in Wt, eNOS \(-\)/, and Akt1 \(-\)/ hearts, before and after 3 days of EPO administration.

**Transthoracic Echocardiography**
Mice were lightly anesthetized with 1.5% isoflurane, and the measurements were made with a 707B 30-MHz ultrasonic probe and recorded on a VEVO 770 System (VisualSonics Inc, Toronto, Canada).

**Statistics**
Grouped data (n = 4 to 6) were expressed as means ± SEM. Statistical analyses were by 2-way or repeated measures ANOVA using StatView (version 5.0.1; SAS Institute, Chicago, Ill). \(P<0.05\) was considered significant.
EPO Responses in Wild-Type, eNOS−/−, and Akt1−/− Mice

Three mouse strains were compared for intactness of erythropoiesis: wild-type (Wt), eNOS−/−, and Akt1−/− (Table 1). Hemoglobin content responded comparably to EPO and to hypoxia over 7 days in Wt and eNOS−/− strains, but lagged behind in the Akt1−/− mice. In Wt mice, EpoR transcript levels in the heart tripled after 3 days of EPO administration and returned to control by day 14 (Figure 2A). EpoR protein at baseline was detected by immunofluorescence in vascular endothelium and cardiomyocyte plasma membranes, but this labeling was sharply decreased by 3 days of EPO administration and returned to control by day 7. EpoR expression, like Akt, was highest in Wt mice at day 3 of EPO administration and return to control by day 7 was examined and Akt1 Ser473 and was found to increase on Akt1-specific antibodies. Extracellular signal-regulated kinase (ERK)1/2 was strongly activated by EPO in Wt mice but was activated minimally in Akt1−/− mice (Figure 2D). EPO failed to increase in Akt1−/− mice after EPO (P<0.05 vs control; n=4).

Figure 3. REE, voluntary exercise, and mitochondrial biogenesis after EPO administration in mice. A, Left, State 3 respiration in control and EPO-treated Wt mouse heart mitochondria with malate/glutamate (open circles) or succinate (closed circles) substrate (P<0.05 before vs after EPO for state 3). A, Right, REE measured from oxygen uptake (VO2) and carbon dioxide production (VCO2) before and after EPO in Wt and Akt1−/− mice (*P<0.05 vs day 0; #P<0.05, Wt vs Akt1). REE failed to increase in Akt1−/− mice after EPO. B, Voluntary exercise on calibrated wheels. Data are averages for days 1 to 3, 4 to 7, and 8 to 14. B, Left, All mice were self-trained by increasing their running distance. B, Right, Running speed increases after EPO (P<0.05). C, Activation of cardiac mitochondrial biogenesis. NRF-1, PGC-1α, and Tfam mRNA levels and mtDNA copy number measured before and after EPO in Wt control mouse hearts (P<0.05 vs control; n=4).
day 3 (Figure 2E). HO-1 was also increased in eNOS\(^{-/-}\) mice but not in Akt1\(^{-/-}\) mice (Figure 2E). EPO thus exploits eNOS in the heart to activate Akt and ERK1/2, and Akt1 acts upstream of ERK1/2 and HO-1.

Respiration, REE, and Voluntary Exercise

EPO did not affect mitochondrial RCR in the heart, but at day 3 after EPO administration, state 3 respiration increased significantly (Figure 3A, left), accompanied by \( \approx 30\% \) increases in REE (Figure 3A, right) at days 7 and 10 \((P<0.05)\). Compared with Wt mice, basal REE was marginally depressed in Akt1\(^{-/-}\) mice \((P=0.06)\) and did not respond to EPO (Figure 3A, right). REE was checked in 2 eNOS\(^{-/-}\) mice and predictably did not respond to EPO, so this experiment was not repeated.

Voluntary exercise in untrained Wt mice was quantified over 2 weeks using ergometer-calibrated running wheels. Typically, most sedentary mice allowed to run ad libitum over this interval will increase distance and running time at constant speed.\(^{23}\) Control and EPO-treated mice demonstrated comparable running distances (Figure 3B), but during the second week, EPO-treated mice spontaneously covered the same distances in less time \((P<0.05);\) Figure 3B, right) but did not significantly increase their total running distance.

Post-EPO Transcriptional Activation of Cardiac Mitochondrial Biogenesis

EPO stimulated the expression of cardiac regulatory genes for mitochondrial biogenesis, including NRF-1, the PGC-1\(\alpha\) coactivator, and mitochondrial transcription factor-A (Tfam) (Figure 3C). NRF-1, PGC-1\(\alpha\), and Tfam mRNA increased 4-fold on day 3 after EPO administration and returned to control by day 14, whereas mtDNA copy number doubled (Figure 3C, right; \(P<0.05\)).

NRF-1, PGC-1\(\alpha\), and Tfam mRNA levels did not respond to EPO in eNOS\(^{-/-}\) or Akt1\(^{-/-}\) mice, and mtDNA copy number was unaffected (Figure 4A and 4B), establishing the requirement for eNOS. SOD2 transcript levels also increased after EPO in Wt but not in eNOS\(^{-/-}\) or Akt1\(^{-/-}\) mice (Figure 4C).

Cardiac Ultrasonography

Cardiac chamber size, wall thickness, and function were evaluated in Wt mice by echocardiography at day 0 and at day 7 after EPO administration. A small increase in intraventric-
We did not formally assess vascular EpoR expression or changes in capillarity in the heart, and although changes in capillary density after EPO have been reported\cite{10}, such responses, if present here, could not be definitively linked to EpoR-mediated regulation of mitochondrial biogenesis at our present level of resolution. In terms of metabolism, the heart in resting mice consumes $\approx 30\%$ of the $O_2$ with a well-defined preference for myocardial $\beta$-oxidation over glucose oxidation. Higher complex II activity would help support the reoxidation of FADH$_2$ (flavin adenine dinucleotide), which requires more $O_2$, but this could quantitatively account for the higher REE only if resting myocardial $O_2$ utilization had doubled. Thus, it is likely that respiration in other organs contributed a modest but unknown amount to the increase in REE.\cite{4,27} Similar constraints apply to the interpretation of the spontaneous exercise data, which demonstrate effectiveness for our EPO protocol, but where the roles of eNOS, Akt1, and changes in skeletal muscle oxidative capacity were not assessed. Skeletal muscle fiber type switching would also be of interest but could reflect the modified submaximal exercise behavior and not a direct effect of EPO, which, among other factors, would depend on EpoR activity in skeletal muscle. Finally, the abundant response by subsarcolemmal mitochondria was not investigated in detail, but these mitochondrial populations do serve different cellular functions and are differently regulated. Overall, however, there is good temporal and quantitative correlation among the molecular, functional, and structural manifestations of cardiac mitochondrial biogenesis after EPO administration.

The therapeutic potential of EPO in acute coronary and cerebral ischemia\cite{28} involves antiapoptotic, antiinflammatory, and angiogenic effects\cite{28,29} for which the prosurvival serine/threonine kinase Akt1/2 is a key effector.\cite{11} Here, Akt1 was required for EPO to stimulate cardiac mitochondrial biogenesis, in keeping with the activating role of Akt/NRF-1 mitochondrial biogenesis.\cite{14,30} The transcriptional program is also activated by eNOS,\cite{15,26,31} and, as an aside, we note that eNOS must be active for EPO to prevent apoptosis and limit experimental cardiac infarct size.\cite{9} EPO-induced mitochondrial biogenesis conforms to eNOS-inducible metabolic gene regulation, eg, by CREB (cAMP-responsive element-binding protein), and the requirement for Akt1 fits its role in growth, metabolism, and cell survival. Also, Akt, eNOS, and HO-1 exhibit an elaborate integration: Akt activates eNOS by calcium-independent phosphorylation,\cite{32} and NO activates phosphatidylinositol 3-kinase, an important Akt regulator, and under some conditions, Akt itself.\cite{33} The Wt and eNOS$^{-/-}$ mice also upregulate HO-1, the latter without new mitochondrial biogenesis, implying that HO-1 is upstream of eNOS in this case.

The physiological responses to EPO are conventionally attributed to hemoglobin biosynthesis and erythropoiesis, which increase red cell mass, and thus hematocrit and arterial $O_2$ content (Ca$_{O_{2}}$).\cite{27,34} In erythroid progenitor cells, EPO is also involved in the regulation of iron uptake through the posttranscriptional induction of transferrin receptor.\cite{35} EPO also directly stimulates the transcriptional and posttranscriptional expression of 5-aminolevulinic acid synthase (ALAS-E), which resides in mitochondria and is the rate-limiting step in heme biosynthesis\cite{36} and necessary for cytochrome assembly.

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**Table 2. Transthoracic Echocardiography Measurements in Wt Mice Before and After EPO**

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pre-EPO</th>
<th>Post-EPO</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW (g)</td>
<td>25.8±0.7</td>
<td>26.4±0.5</td>
<td>0.46</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.0±0.2</td>
<td>3.2±0.3</td>
<td>0.24</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.5±0.2</td>
<td>1.8±0.3</td>
<td>0.32</td>
</tr>
<tr>
<td>IVSTs (mm)</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
<td>0.59</td>
</tr>
<tr>
<td>IVSTD (mm)</td>
<td>0.8±0.1</td>
<td>1.0±0.1</td>
<td>0.03*</td>
</tr>
<tr>
<td>PWTD (mm)</td>
<td>1.4±0.2</td>
<td>1.3±0.2</td>
<td>0.76</td>
</tr>
<tr>
<td>PWTS (mm)</td>
<td>1.9±0.9</td>
<td>1.5±0.2</td>
<td>0.26</td>
</tr>
<tr>
<td>FS (mm)</td>
<td>0.51±0.1</td>
<td>0.45±0.1</td>
<td>0.61</td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>458±27</td>
<td>484±6</td>
<td>0.44</td>
</tr>
<tr>
<td>LVM mass (mg)</td>
<td>135±11</td>
<td>121±5</td>
<td>0.42</td>
</tr>
</tbody>
</table>

**Note:** BW indicates body weight; FS, fractional shortening; HR, heart rate (bpm); IVSTs, interventricular septal thickness in diastole (mm); IVSTD, interventricular septal thickness in systole (mm); LVEDD, left ventricular end-diastolic dimension (mm); LVESD, left ventricular end systolic dimension (mm); PWTD, posterior wall thickness in diastole (mm); PWTS, posterior wall thickness in systole (mm); LVMi, left ventricular mass index ($[(LV$ external $DV$ diameter diameter$)^2]−[LV$ end-diastolic dimension$]^3])×1.055$ (mg). Values are means±SEM for $n=4$.

**Discussion**

The novel aspects of this work disclose that EPO stimulates cardiac mitochondrial proliferation through a highly regulated, receptor-mediated, eNOS/Akt1-dependent cascade that activates the transcriptional program of mitochondrial biogenesis. This activity occurs over approximately 3 to 7 days in mice and leads to a higher state 3 respiration, REE, and running speed in advance of a rising hematocrit, which increases at 1% to 2% per day. Although the EPO response requires eNOS,\cite{26} the sites of microvascular NO production and NO signaling on a paracrine or a cell-by-cell basis were not evaluated; however, we did detect an unexplained increase after EPO in EpoR receptor density in the heart and in cardiomyocytes\cite{27} but found no short-term structural evidence of left ventricular hypertrophy.

The interpretation of these studies has some limitations. We did not formally assess vascular EpoR expression or changes in capillarity in the heart, and although changes in...
Clinically, EPO treatment mitigates the anemia of chronic kidney disease, although EPO increases mortality in cancer patients and when the target hemoglobin is set too high. In human athletes, EPO enhances endurance by improving maximum O₂ uptake in direct relation to hematocrit; however, a role for EPO in maintaining cardiac function in hypoxia and anemia, the main settings for its production, although long suspected, has been unconfirmed.

In the context of hypoxia, the regulation of cardiac mitochondrial density by EPO implies that a distinct spatial and functional arrangement of the organelles is necessary for optimal aerobic work performance at steady-state convective oxygen transport (cardiac output times CaO₂) as hematocrit, the principal determinant of blood viscosity, increases. Because blood viscosity is governed by hematocrit (at constant shear rate), the inherent matching of cardiac mitochondrial mass to erythrocyte O₂ carrying capacity, despite the heart’s normally high oxygen extraction ratio, and barring limitations in coronary blood flow, would serve to protect cardiac oxidative phosphorylation and therefore maintain peripheral tissue O₂ delivery during hypoxia. By comparison, in anemia of nonrenal origin, the cardiac structure–function implications are less clear because endogenous EPO secretion tends to be elevated and it is not known whether mitochondrial volume density is affected.

In conclusion, cardiac EpoR activation after EPO administration activates mitochondrial biogenesis in normal mice before significant increases in circulating hemoglobin concentration. Ultimately, the physiological increase in mitochondrial density serves aerobic cardiac performance at a higher blood viscosity, but the effectiveness of this would be contingent on a higher myocardial O₂ uptake stemming from an adjustment in aerobic capacity for contractile function designed for use in hypoxia. In addition to the new physiological role for EPO, our findings have latent implications for the treatment of patients with ischemic cardiomyopathy and anemia, often associated with chronic kidney disease, who may be candidates for stabilization of ventricular and renal function by the administration of erythropoietic agents but who may also have myocardium at risk under conditions of an elevated cardiac O₂ demand.

**Acknowledgments**
We thank Martha Salinas and Craig Marshall for technical assistance and G. Allen Johnson, PhD, for support of the echocardiography.

**Sources of Funding**
This work was supported by NIH grants R01 AI0664789 and HL 090679 (to C.A.P.).

**Figure 5. EpoR requirement for EPO-mediated nuclear NRF-1 accumulation in cardiomyocytes.**
H9C2 rat cardiomyocytes were transfected with scrambled (Scr) or EpoR siRNA followed by EPO treatment (1000 U/mL). After 24 hours, fluorescence microscopy was performed using anti-NRF-1 (red) and EpoR (green). A through C, Scrambled RNA pre-EPO. D through F, Scrambled RNA post-EPO. G through I, Post-EPO plus siRNA. Nuclear NRF-1 translocation by EPO requires EpoR expression.
Disclosures
The research described in this article has been reviewed by the Health Effects and Environmental Research Laboratory, US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents reflect the views and the policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

References
What Is Known?

- The renal hormone erythropoietin (EPO) regulates the production of RBCs and acts to protect the heart, brain, and other tissues from certain types of injuries, such as ischemia/reperfusion, but by unknown mechanisms.
- EPO supplementation also improves exercise performance by increasing the supply of RBCs and possibly by improving capillary density.
- The EPO receptor is expressed in several types of nonerythroid cells, but the reasons for this are poorly understood.

What New Information Does This Article Contribute?

- The administration of EPO stimulates the heart to produce new mitochondria through EPO receptor–dependent activation of the transcriptional program of mitochondrial biogenesis.
- EPO induces mitochondrial biogenesis in the heart before circulating RBC mass increases and leads to increases in both resting energy expenditure and running speed in mice.
- The mechanism of EPO-dependent mitochondrial biogenesis requires endothelial NO synthase and the prosurvival kinase Akt-1.

The renal hormone EPO is used clinically to treat anemia in patients with chronic cardiac and kidney diseases; however, EPO may offer benefits beyond an improvement in hematocrit. Here, we investigated whether EPO influences the heart’s capacity to produce mitochondria in mice. After 3 days of EPO administration, mice developed sharp increases in mtDNA content and mitochondrial density throughout the myocardium, which peaked by 7 days, preceding and surpassing effects on hepatic renal and skeletal muscle and occurring before significant increases in blood hemoglobin content. This response was associated with increases in peak mitochondrial respiration rates, resting energy expenditure, and spontaneous running speed. EPO activated cardiac mitochondrial biogenesis through the endothelial NO synthase and the Akt1 prosurvival kinase by enhancing gene expression for the activators NRF-1 (nuclear respiratory factor-1), PGC-1α (peroxisome proliferator-activated receptor γ coactivator 1α) and mitochondrial transcription factor-A. An EPO receptor requirement was demonstrated by receptor-silencing in cardiomyocytes, which blocked the nuclear translocation of NRF-1 mediated by EPO. These new findings indicate a physiological and protective role for EPO, acting through its cell surface receptor and NO-Akt1 signal transduction, in matching the mitochondrial mass in the heart to the body’s oxygen transport capacity as the circulating erythrocyte mass expands.
Erythropoietin Activates Mitochondrial Biogenesis and Couples Red Cell Mass to Mitochondrial Mass in the Heart
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Supplement Material

Detailed Materials and Methods

*Mice.* All animal studies were approved by the Duke University Institutional Animal Care and Use Committee and conducted in 10-15 week old male C57BL/6J, Akt1−/− and eNOS−/− mice purchased from Jackson Laboratory (Bar Harbor, ME) or in genetically-engineered mitochondrial GFP (green fluorescent protein) reporter mice obtained from Tokyo Metropolitan Institute of Medical Science (Tokyo, Japan), and bred in our vivarium \(^1\). Mice were injected subcutaneously with human recombinant EPO (4000 U/kg; Amgen, Thousand Oaks, CA) or an equal volume of 0.9% NaCl once daily for three consecutive days and tissues removed under general anesthesia at the indicated times.

*Histology and fluorescence microscopy.* Fresh tissues were fixed in 10% formalin, paraffin-embedded and sectioned at 5 µm, mounted on slides, de-paraffinized, treated with 0.1% saponin, and washed in PBS. Slides were prepared with SlowFade antifade kit (Invitrogen, Carlsbad, CA) and laser-scanning confocal microscopy was performed on a Zeiss LSM 410 microscope (MicroImaging, Inc., Thornwood, NY). Fluorescence images were acquired on a Nikon microscope through a 520 nm filter and fluorescence quantified using computer software (Nikon NIS-Element F 3.0).

*Mitochondria studies and immunobLOTS.* Cardiac proteins were resolved by SDS-PAGE, then transferred to polyvinylidene difluoride membranes, and probed with polyclonal Akt and phospho-Akt antibodies (sc-8312 and sc-135651), Erk 1,2, and phospho-Erk 1,2 (pErk), and HO-1 primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Akt1-specific antibodies were also used for negative control blots in Akt1−/− mice (Akt1; sc-81434 and phospho-Akt1; sc-81433; Ser 473). After application of primary antibody, the membranes were washed and incubated with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies (Santa Cruz or Jackson) and the membranes developed in ECL (Santa Cruz) and quantified on digitized images in the mid-dynamic range. Protein loading was confirmed by stripping the membranes and probing for tubulin or β-actin (Sigma, St. Louis, MO). At least four samples were used for densitometry measurements. All proteins were checked pre and at 3, 7 and 14 days post-EPO, and where there was no response to EPO, only day 3 data were displayed in the histograms.

Pooled cardiac mitochondria were prepared by discontinuous Percoll gradient centrifugation and 1-2 mg of mitochondrial protein was suspended respiration buffer in water-jacketed chambers at 35°C \(^2\). States 2, 4 and 3 (ADP-supplemented) respiratory rates were measured with succinate (5 mM) or malate + glutamate (2.5 mM) using calibrated Clark mini-electrodes (Diamond General, Ann Arbor, MI) \(^3\).

*Nucleic acids.* Cytoplasmic RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) and cDNA synthesized using the SUPERSCRIPT System Kit (Invitrogen). Cardiac mtDNA was isolated using NaI kits (Wako, Tokyo). Mouse-specific primers and probes were designed with Primer Express (Applied Biosystems, Branchburg, NJ), and RT-PCR performed as published \(^4\) RNA samples (1µg) were reverse-transcribed (in 20 µl) using Moloney murine leukemia virus reverse transcriptase (180 units; Promega, Madison, WI) in a buffer containing random hexamer primers, dNTPs, and ribonuclease inhibitor RNasin (Promega). The transcripts were amplified in triplicate using gene-specific primers \(^4\,\,5\) and quantified by densitometry with normalization to
18S rRNA or GAPDH (Bio-Rad, Hercules, CA). MtDNA copy number was determined by PCR as reported 6.

Metabolic measurements. Steady-state O\textsubscript{2} consumption and CO\textsubscript{2} production (\(\bar{V}O_2\) and \(\bar{V}CO_2\)) were measured in resting mice pre-acclimated to individual metabolic chambers at constant ambient temperature (25\textdegree C) at the same time of day 7. After a stabilization period, the mouse’s expired gas was collected for 6-8 min at a calibrated flow rate. The O\textsubscript{2} and CO\textsubscript{2} gas concentrations were measured on a calibrated gas chromatograph (Model 3800; Varian, Palo Alto, CA) and the \(\bar{V}O_2\) and \(\bar{V}CO_2\) were computed with standard formulae including temperature corrections. Resting energy expenditure (REE) was calculated using the modified Weir formula and expressed in kcal/d 8.

Voluntary exercise. Mice were supplied ad libitum with food and water, and housed individually in cages containing rodent exercise wheels adapted from 9. The system consisted of an 11.5-cm diameter wheel with a 5.0-cm-wide running surface (Model 6208, Petsmart, Phoenix, AZ) equipped with a digital magnetic counter activated by wheel rotation. To minimize the variability in the time spent running at the start of the study, individual mice were initially observed for 72 hours, and then assigned randomly to groups that were well-matched for running times. One group received EPO and the other NaCl for three consecutive days, and over the next two weeks, running duration, running speed, and distance in kilometers were recorded daily for each mouse.

Transthoracic echocardiography. Mice were lightly anesthetized with 1.5% isofluorane and the measurements made with a 707B 30 MHz ultrasonic probe and recorded on a VEVO 770 System (VisualSonics, Inc., Toronto).

Statistics. Data from 4 to 6 mice per group were expressed as means ± SEM. Statistical analyses was by two-way or repeated measures ANOVA using StatView (SAS Institute, 5.0.1; Chicago, IL). \(P\leq 0.05\) was considered significant.
References


Online Figure I: Cardiac mitochondria pre- and post-EPO (day 7) in mitochondrial reporter mice. Pre-EPO sections are shown in panels A and B (100 and 1,000 magnification scale bars are 50 and 5 microns, respectively). Comparable images are shown in panels C and D at post-EPO day 7.
Online Figure II: Mitochondrial citrate synthase expression in mitochondrial reporter mice. Reporter mice expressing the GPF mitochondrial localization tag received EPO (4,000 U/kg/d) for three days and hearts were compared by fluorescence microscopy pre- and post-EPO. These tissues were then labeled with anti-citrate synthase (red fluorescence), and the images merged to demonstrate co-localization (yellow-orange). Citrate synthase staining matched the distribution of GFP, and was present with light diffuse staining pre-EPO, that increased nearly uniformly post-EPO at days 3 and 7. Scale bars are 5 microns.