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Erythropoietin Changes Contractility, cAMP, and Nitrite Levels of Isolated Rat Hearts

Ziya KAYGISIZ[1], Nilufer ERKASAP[1], Nuray YAZIHAN[3], Kemal SAYAR[4], Haluk ATAOGLU[5], Ruhi UYAR[1], and Murat IKIZLER[2]

[1]Department of Physiology and [2]Cardiovascular Surgery, Eskiehir Osmangazi University, Medical Faculty, 26480, Eskişehir, Turkey; and [3]Physiopathology, [4]Pharmacology, and [5]Microbiology – Molecular Biology Research and Development Unit, Ankara University, Medical Faculty, Morfoloji, 06100, Ankara, Turkey

Abstract: There is enough evidence that erythropoietin (EPO) may be involved in cardiovascular function. Therefore we have investigated the possible effects of EPO on left ventricular developed pressure, +dP/dt_{max}, heart rate, tissue cAMP, and nitrite levels. Isolated rat hearts were perfused under constant flow (10 ml/min) conditions with modified Krebs-Henseleit solution and recombinant human erythropoietin at doses of 100, 200, 500, and 1,000 IU/kg was administered as bolus injections. EPO at 100 IU/kg decreased, but higher doses (500 and 1,000 IU/kg) raised the developed pressure and +dP/dt_{max}. However, it did not affect heart rate or coronary perfusion pressure when all the respective doses were applied. EPO at 100 IU/kg increased nitrite, and at 1,000 IU/kg it raised cAMP. Our results suggest that EPO may produce dose-dependently negative and positive inotropic effects on myocardial contractility in isolated rat hearts. NO and cAMP may be involved in negative and positive inotropic effects of EPO, respectively.

Key words: erythropoietin, heart, contractility, cAMP, nitrite.

Erythropoietin (EPO), a glycoprotein hormone produced mainly in the kidney [1], is essential for erythropoiesis [2]. The EPO receptor is a member of the cytokine receptor superfamily [3] and is expressed in various cells, such as erythroid precursors [4], endothelial [5], kidney [6], neuronal [7], smooth muscle cells, and cardiomyocytes [2]. EPO increases neonatal rat cardiomyocyte proliferation [8] and protects the heart from ischemia-reperfusion injury [9]. It also protects cardiac myocytes from hypoxia-induced apoptosis [10] and has mitogenic [11] and angiogenic [12] effects on vascular endothelium.

It has been shown that recombinant erythropoietin (rHuEpo) is effective for the treatment of anemia associated with end-stage renal disease [13]. The treatment of renal anemia with EPO enhances cardiac output [14] and improves cardiac exercise performance [2]. EPO (10–200 IU/ml) induces a direct vasoconstrictor effect on renal resistance vessels [15], and intravenous EPO administration in hemodialysis patients causes an increase in plasma endothelin and blood pressure [16]. These findings suggest that EPO may play a role in the regulation of cardiovascular function.

EPO-induced increase in intracellular cAMP has been indicated in mouse erythroleukemia SKT6 cells [17] and erythroblasts [18]. Cardiac myocytes and vascular endothelial cells produce nitric oxide (NO) via both constitutive and inducible isoforms of NO synthase [19]. NO stimulates cGMP formation, and EPO administration also increases plasma cGMP in rats [20]. It is possible that the effect of EPO on cardiac cell contractility might be mediated either alone by cAMP or by NO, or by both together.

Since the effect of EPO on myocardial contractility, heart rate, and coronary perfusion pressure and the roles of cAMP and NO in contractile responses to EPO have not been investigated, we have decided to study these parameters on isolated perfused rat hearts.

MATERIALS AND METHODS

Langendorff perfusion. Sprague-Dawley rats of either sex weighing 300–400 g were used. All procedures were applied in accordance with the Guiding Principles in the Care and Use of Animals. One hour after the administration of 1,000 IU heparin i.p., the chest was opened under light ether anesthesia, and the heart was excised rapidly and then placed into an ice-cold modified Krebs-Henseleit solution (mKHs) until contractions ceased. After the heart was cleansed of surrounding tissues, the aorta was immediately tied to a stainless steel perfusion cannula, and the heart was perfused retrogradely by the nonrecirculating Langendorff technique [21]. The pulmonary artery was incised to facilitate a complete coronary drainage in the ventricles. The perfusion solution was daily prepared

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Correspondence should be addressed to: Ziya Kaygisiz, Eskiehir Osmangazi University, Medical Faculty, Department of Physiology, 26480, Eskisehir, Turkey. Tel: +90 222 239 29 79/4575, Fax: +90 222 239 37 72, E-mail: ziyak@ogu.edu.tr

mKHs with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11. The perfusion solution was continuously oxygenated with 95% O_2 and 5% CO_2, using a disposable infant oxygenator (Dideco Masterflo, Mirandola), and pH of the solution was 7.4. The temperature was continuously measured and maintained at 37°C. The hearts were perfused under constant flow condition (10 ml/min) using an infusion pump (LifeCare^R Pump, Abbott/Shaw, Chicago).

Measurement of hemodynamic parameters. The myocardial contractile force was measured according to the method described previously [22]. A liquid-filled balloon was connected to a pressure transducer (Transpack II, Abbott, Bedford, MA) and inserted into the left ventricle via the mitral valve. The diastolic balloon pressure was maintained at 10 mmHg. Left ventricular developed pressure (sistolic pressure-diastolic pressure), $+dP/dt_{max}$, and heart rate from the tracings of the left ventricular pressure were measured by software (Biopac System, Santa Barbara, CA). The coronary perfusion pressure was measured by attaching a side arm of the aortic cannula to a pressure transducer (TB 200T, Nihon Kohden, Tokyo), as described earlier [23], and then recorded on a polygraph (RM 6000, Nihon Kohden). The hearts were equilibrated for 30 min to establish a stable baseline. rHuEpo (Epoetin β, Roche) was injected in doses of 100 (n = 9), 200 (n = 8), 500 (n = 8), and 1,000 IU/kg (n = 11) in a bolus fashion into the perfusate, 2 cm proximal to the aortic cannula. Each dose was applied to a separate group of hearts. Our preliminary experiments indicated that no modification in cardiac or coronary parameters could be detected after a bolus administration of EPO-free physiological salt solution. During the measurement of hemodynamic parameters 0.2 mM 3-isobutly-1-methyl-xantine (IBMX), a cyclic nucleotide phosphodiesterase inhibitor was infused for 30 min to prevent a breakdown of cAMP accumulation [19].

Measurement of nitrite. Nitrite was quantitated in heart tissue by using the diazotization method, which depends on the diazotization of nitrite with an aromatic amine (sulphonamide) and the formation of a colorful azo derivative with *N*-(1-Naftil) etilendiamine (Griess reaction). The tissue samples were homogenized (Polymix, Switzerland) and centrifuged (Sigma) in 0.1 M phosphate-buffered saline pH 7.4 at 10,000 rcf, 10 min. The absorbance of supernatants was measured spectrophotometrically at 545–555 nm wavelength. This method is sensitive to 5×10^{-8}–5×10^{-5} M nitrite levels [24].

Measurement of cAMP. The tissue samples were frozen at –80°C until measurement. The ventricles were then dissected and homogenized in cold 0.1 N HCl (1:5) and centrifuged at 10,000 rcf, 10 min. Briefly, 10 µl of the samples were used to measure cAMP level. Cyclic AMP accumulation was measured in the supernatants as described previously, with some modifications [25]. Cyclic AMP was determined by radioimmunoassay with the acetylation protocol. High-affinity rabbit anti-cAMP antibodies were raised in our laboratory by using BSA-conjugated cAMP. Succinyl-cAMP tyrosine methylester (ScAMP-TME) was iodinated by the chloramine-T method. Mono- and di-iodo ScAMP-TME, which were used as tracer ligands for radioimmunoassay, were purified by gel-filtration chromatography (Sephadex G 25 superfine), equilibrated, and eluted with 1 M sodium acetate, pH 5).

Statistical analysis. Values are given as mean ± SEM. The values of the developed pressure, heart rate, and perfusion pressure obtained before the addition of EPO were taken as controls. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by the Tukey-HSD multiple comparisons test. One-way analysis of variance (ANOVA) was applied to cAMP and nitrite. A p value of less than 0.05 was considered significant.

RESULTS

Developed pressure

The effect of different doses of EPO on the developed pressure is shown in Fig. 1. The bolus injection of EPO at 100 IU/kg (n = 9) reduced the developed pressure from

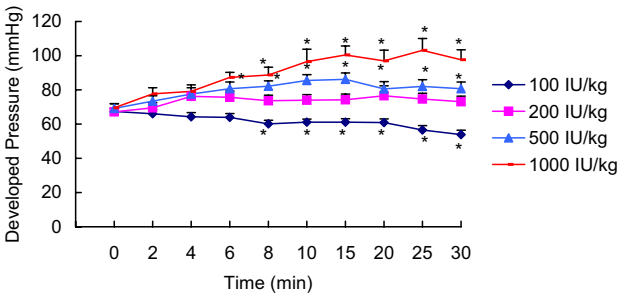


Fig. 1. Time course of the effects of EPO on the developed pressure. Time 0 represents control values. * Significantly different from the respective control ($p < 0.001$).

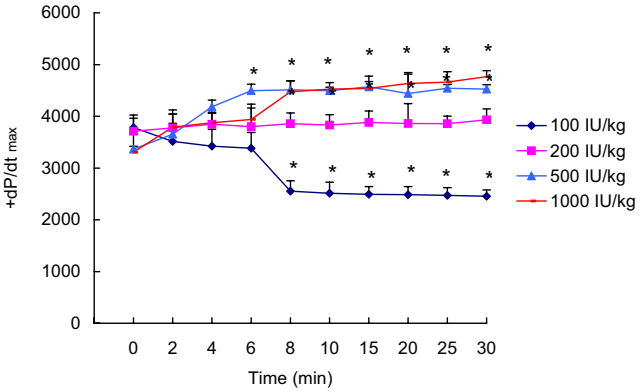


Fig. 2. Time course of the effects of EPO on the $+dP/dt_{max}$. Time 0 represents control values. * Significantly different from the respective control ($p < 0.05$).

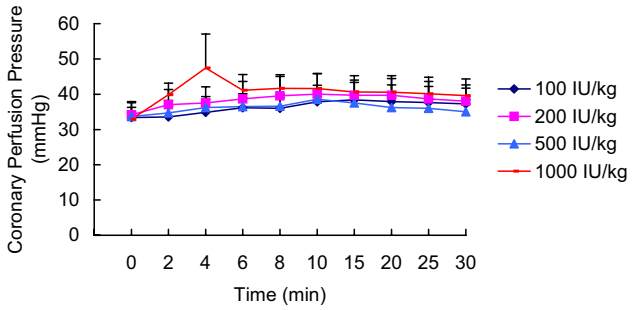


Fig. 3. The action of EPO on the coronary perfusion pressure.

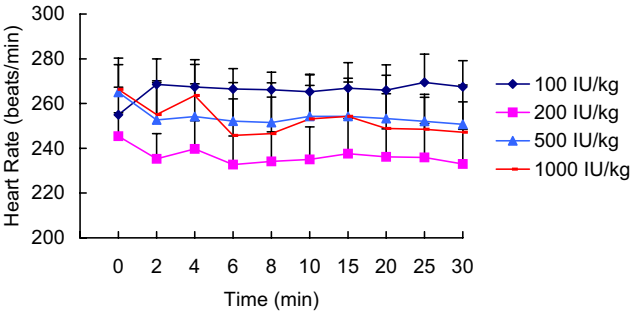


Fig. 4. The effect of EPO on the heart rate.

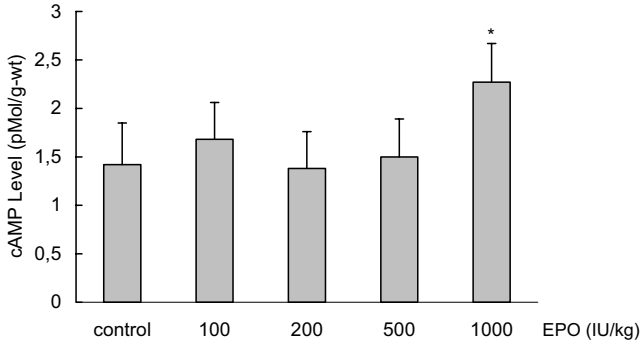


Fig. 5. Cardiac tissue cAMP levels. * represents the difference from the other groups ($p < 0.05$).

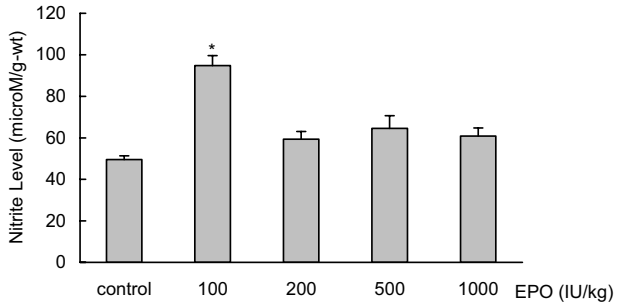


Fig. 6. Cardiac tissue nitrite levels. * represents the difference from the other groups ($p < 0.05$).

67.32 ± 1.8 to 53.9 ± 2.48 mmHg ($p < 0.001$). On the other hand, EPO given at 200 IU/kg ($n = 7$) slightly increased the developed pressure from 67.12 ± 2.09 to 76.58 ± 5.8 mmHg ($p > 0.05$). However, EPO at 500 IU/kg increased the developed pressure from 69.14 ± 2.83 to 86.16 ± 3.76 mmHg ($p < 0.001$, $n = 7$), and at 1,000 IU/kg from 69.54 ± 2.3 to 103.16 ± 6.85 mmHg ($p < 0.001$, $n = 11$).

+dP/dt_{max}

EPO at 100 IU/kg ($n = 9$) decreased ($p < 0.05$) and EPO at 200 IU/kg ($n = 7$) slightly increased the +dP/dt_{max}. However, EPO at 500 ($n = 7$) and at 1000 IU/kg ($n = 11$) significantly increased ($p < 0.05$) the +dP/dt values (Fig. 2).

Heart rate

EPO at the doses used in the present study had no significant effect on either the coronary perfusion pressure ($n = 8$, Fig. 3) or the heart rate ($n = 8$, Fig. 4).

Biochemical results

EPO injection at a dose of 100 IU/kg ($n = 9$) caused a slight increase in the tissue cAMP. EPO at a dose of 200 IU/kg ($n = 7$) slightly decreased and at a dose of 500 IU/kg ($n = 8$) slightly increased cAMP level ($p > 0.05$ for both). However, EPO at a dose of 1000 IU/kg ($n = 11$) significantly raised cAMP from 1.42 ± 0.2 to 2.27 ± 0.4 pMol/g-wt ($p < 0.05$, Fig. 5).

EPO at 100 IU/kg ($n = 9$) elevated the tissue nitrite level from 45.97 ± 16.7 to 94.8 ± 17 μM/g-wt ($p < 0.05$). EPO at 200 ($n = 7$), 500 ($n = 8$), and 1,000 IU/kg ($n = 11$) produced a small but not significant increase in the nitrite level (Fig. 6).

DISCUSSION

The present study is the first to apply different doses of EPO in isolated rat hearts. We have found that EPO has different contractile effects at different doses. A bolus injection of EPO (100 IU/kg) significantly elevated nitrite, a stable end product of NO oxidation, and decreased the developed pressure, an index of contractility and +dP/dt_{max}. However, it slightly increased cAMP content. In vitro studies have shown that 0.1–20 IU/ml EPO increased NO release from rat renal arcuate arteries and human umbilical vein endothelial cells in culture [26]. Our dose is in the range of 0.1–20 IU/ml. EPO therapy also increased plasma cGMP in chronic-renal-failure patients on hemodialysis [27]. The negative inotropic effect of EPO may be mediated by a cAMP-independent mechanism because the increased cAMP levels increase myocardial contractility [19]. NO might be involved in the negative inotropic effect of EPO, since NO reduces calcium current into myocytes [28–30]. Calcium current reduction leads to a decrease in contractile force by reducing intracellular calcium. The finding that NO attenuates cardiac contractility [19] is consistent with our result. Moreover, cGMP prevents the positive inotropic effect of cAMP

[31], and NO inhibits cAMP production in cardiac myocytes via a guanylyl cyclase–independent mechanism [19]. These results may explain our contractile force and cAMP results with 100 IU/kg of EPO. Studies with nitric oxide donors showed that the effects of NO on myocardial function is dose dependent; a lower concentration usually results in an increase in contraction [32].

We have observed that 200 IU/kg EPO did not significantly affect the developed pressure and the $+dP/dt_{max}$, but 500 IU/kg EPO enhanced the developed pressure and the $+dP/dt_{max}$. This dose induced an insignificant increase in cAMP; 1,000 IU/kg EPO increased the developed pressure, $+dP/dt_{max}$, and cAMP level. The increase in cAMP observed with EPO treatment was prominent. Thus cAMP may be involved in the increase of contractile force. It has been found that EPO enhances myocardial contractility in mice, and contractility shows a positive correlation with plasma EPO [33]. These *in vivo* findings support the EPO-induced positive inotropic effects observed in the present study. It has been reported that EPO at high doses (> or =20 IU/ml) elevates and at lower doses (<20 IU/ml) does not affect intracellular Ca^{2+} content in cultured smooth-muscle cells [34]. EPO doses higher than 20 IU/ml correspond to our >200 IU/kg. Similarly, it is possible that EPO at high doses increases intracellular Ca^{2+}. Our results indicate that an EPO-induced increase in myocardial contraction may be mediated by cAMP. In cardiac myocytes, cAMP-dependent protein kinase (PKA) phosphorylates phospholamban (PLB) and sarcoplasmic reticulum (SR) calcium release channel/ryanodine receptor (RyR) [35]. Phosphorylation of PLB increases SR Ca^{2+} uptake [36] by altering SR Ca^{2+} ATPase activity [35]. RyR phosphorylation enhances the open time of the RyR [37]. Furthermore, PKA has been reported to increase Ca^{2+} influx through the voltage-gated Ca^{2+} channel. The Ca^{2+} current induces Ca^{2+} release from SR and increases Ca^{2+} in cytoplasm and SR [38]. It has been reported that EPO (20 IU/ml) raises the levels of intracellular inositol 1,4,5-triphosphate (IP_3) in contractile rat glomerular mesangial cells [39]. IP_3 may also play a role in EPO-induced positive inotropic effect on heart muscle. Further studies are necessary to clarify the effect of EPO on cardiac contractile force. In our study, high doses of EPO did not significantly affect NO level. Furthermore, single bolus injections of 400 IU/kg EPO raised plasma cGMP [20], which is a second messenger of NO. In that study, only 400 IU/kg was used. Further studies on the relationship between EPO and NO may be explanatory. We found that 500 and 1,000 IU/kg EPO increased contractility. It has been reported that a higher concentration of NO results in a decrease in contraction [32].

We have found that EPO did not alter the coronary perfusion pressure. Our results are in agreement with the results obtained from rat mesenteric and renal arcuate arteries [40], rabbit aorta [41], rat thoracic aorta [42], aortic rings [43], and rat mesenteric arteries and human subcutaneous arteries [44]. These studies did not indicate the presence of a vasoconstrictor effect of EPO.

EPO therapy decreased heart rate in children with end-stage renal disease [45]; however, it had no significant effect on heart rates in patients with orthostatic tachycardia [46]. EPO also did not significantly alter heart rates in rats *in vivo* [20]. In isolated hearts, the action of EPO on heart rate has not been studied. Our results indicate that the different doses of EPO did not affect heart rate. We believe that additional studies are needed to determine the possible effect of EPO on heart rate.

In conclusion, our results suggest that EPO may induce different effects on cardiac contractility at different doses. A negative inotropic effect might be mediated by increased NO, and a positive inotropic effect might be mediated by increased cAMP. EPO at a low dose may reduce contractility and increase NO, but at a high dose may increase both cAMP level and myocardial contractility. In consideration of this effect, EPO may enhance stroke volume and thus increase the blood volume delivered to tissues. In patients with chronic renal insufficiency, impaired myocardial function induced by renal anemia has been reported [47]. Therefore we propose that EPO at high doses may improve cardiac function in renal anemia.

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