Published in final edited form as:

J Mol Cell Cardiol. 2012 January ; 52(1): 256–263. doi:10.1016/j.yjmcc.2011.10.005.

Erythropoietin induces positive inotropic and lusitropic effects in murine and human myocardium

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Abstract

Initial clinical studies indicate a potential beneficial effect of erythropoietin (EPO) in patients with anemia and heart failure. Here, we investigate the direct contractile effects of erythropoietin on myocardial tissue. Treatment with EPO (50 U/ml) using excitable murine and human left ventricular muscle preparations resulted in a 37% and 62% increase in twitch tension, respectively ($P < 0.05$). Isolated murine cardiomyocytes exposed to EPO demonstrated a 41% increase in peak sarcomere shortening ($P = 0.012$). Using compounds that specifically stimulate a non-erythropoietic EPO receptor yielded similar increases in contractile dynamics. Cardiomyocyte Ca^{2+} dynamics showed an 18% increase in peak calcium in EPO treated cardiomyocytes over controls ($P = 0.03$). Studies in muscle strips skinned after EPO treatment demonstrated a phosphorylation dependant increase in the viscous modulus as well as an increase in oscillatory work. The EPO mediated increase in peak sarcomere shortening was abrogated by PI3-K blockade via wortmannin and by non-isozyne specific PKC blockade by chelerythrine. Finally, EPO treatment resulted in an increase in PKC ϵ in the particulate cellular fraction, indicating activation of this isoform. EPO exhibits direct positive inotropic and lusitropic effects in cardiomyocytes and ventricular muscle preparation. These effects are mediated through PI3-K and PKC ϵ isoform signaling to directly affect both calcium release dynamics and myofilament function.

Keywords

heart failure; contractility; myofilament; erythropoietin

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Disclosures

None

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Introduction

The potential benefit of using erythropoietin (EPO) to treat the anemia associated with chronic heart failure (CHF) has sparked a growing interest in studying the mechanisms by which EPO may exert a beneficial effect in patients with cardiovascular disease. It is now recognized that many tissues, including the myocardium, express surface membrane EPO receptors. As demonstrated in animal models, EPO has pleiotropic effects in the heart, including anti-apoptotic [1], angiogenic [2], and cytoprotective effects against ischemia-reperfusion injury [3]. An increase in left ventricular ejection fraction has been demonstrated with EPO treatment in a small trial of patients with anemia and CHF [4]. However, it is not clear if changes in cardiac performance are the result of a direct effect on contractility, are secondary to one or more of the factors mentioned above, or simply reflect correction of anemia.

Interestingly, the erythropoietic and tissue protective effects of EPO may be mediated by different receptor complexes [5]. Whereas EPO stimulates red cell progenitors by binding to an EPO receptor homodimer, a heterodimer receptor complex composed of the EPO receptor and the common β receptor, also known as CD131 (not to be confused with the β -adrenergic receptor) has been shown to be tissue protective [6]. In the present study, we tested the hypothesis that EPO directly modulates cardiac contractility through an EPO receptor in both murine and human myocardium.

Materials and methods

Expanded methods are described in an online Data Supplement

All experiments in mice were performed according to protocols approved by the University of Vermont Institutional Animal Care and Use Committee. Human epicardial biopsies were obtained at the time of cardiac surgery as previously described [7]. Patient consent was obtained prior to surgery, in accordance with the Committees on Human Research at the University of Vermont

Excitable Human and Murine Left Ventricular Muscle Strip Experiments

Excitable strips were dissected and mounted on an isometric force transducer. Forces were normalized by muscle cross-sectional area. After the contraction reached a steady state (minimum of 5 minutes), 50 U/mL of human recombinant EPO (Epoetin Alfa, Amgen) was added to the circulating modified Ringers solution. Contractile parameters were determined at baseline, and at 5, 10, 15 and 20 minutes after the addition of 50 U/ml of EPO.

Isolated Murine Myocyte Experiments

Murine ventricular cardiomyocytes were obtained as described previously [8]. Cardiomyocyte aliquots from the same heart were incubated with either an incubation buffer or an incubation buffer plus 50 U/ml of EPO for 90 minutes at room temperature before recording sarcomere dynamics or intracellular Ca^{2+} transients (performed at 37° C). For dose-ranging studies cardiomyocytes were incubated with 0, 0.1, 1, 10 or 50 U/ml of EPO.

In separate experiments, cells were incubated with either carbamylated EPO (CEPO, 0.5 mg/ml) or pyroglutamate helix B surface peptide (termed ARA290, 16 nmol/L) following the above protocol. These compounds have been shown to selectively bind the heterodimeric EPO receptor [5,9] and were generously provided by Warren Pharmaceuticals, Ossining, NY.

For inhibitor experiments, 100 nmol/L of the phosphatidylinositol-3-kinase inhibitor wortmannin, 5 μ mol/L of the non-isozyme specific PKC inhibitor chelerythrine or 100 nmol/L of the Ca^{2+} -dependant PKC isozyme inhibitor Gö6976 were added to the cardiomyocytes exposed to 50 U/ml of EPO (90 minute incubation).

Sarcomere shortening dynamics were measured in isolated cardiomyocytes at 37°C as previously described [10]. Cardiomyocytes were field stimulated by platinum electrodes at 8 Hz with stimulus duration of 0.5 ms and voltage of 1.5 times stimulation threshold. Measurement of cardiomyocyte intracellular Ca^{2+} dynamics was performed following incubation with EPO (as described above) and cardiomyocyte loading with 3 μ mol/L fura-2AM (15 minutes at room temperature).

Studies in Skinned Murine Cardiac Strips

Left ventricular skinned myocardial strips were studied as previously described [11]. Mouse papillary muscles were incubated in either control solution (95% O_2 -5% CO_2 bubbled Krebs solution with 30 mmol/L BDM) or control solution plus 50 U/mL EPO at room temperature for 90 minutes prior to demembration. At the time of study, 4 muscle strips, which had been exposed to EPO during incubation, were exposed to 6 U/mL alkaline phosphatase for 10 minutes. Strips were calcium activated from pCa 8.0 to pCa 4.8 and isometric tension was recorded. Sinusoidal length perturbations of amplitude 0.125% strip length were applied at 0.125–250 Hz [11]. The portion of the tension response which was in-phase with the length perturbation provided the elastic modulus; the out-of-phase portion of the tension response provided the viscous modulus.

Myocyte Fractionation and Western Blotting of Murine Myocardium

Freshly dissected myocardial strips were treated as above with 50 U/ml EPO for 90 min and compared to control strips that were treated similarly with the exception of EPO. Cytosolic and particulate cellular fractions were isolated as described [12]. Western blotting of the fractions was conducted as previously reported [13] using the primary antibodies anti-PKC ϵ (Millipore; 4 μ g/mL) and anti-PKC δ (Calbiochem; 1:145 dilution). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control employing a monoclonal anti-GAPDH antibody (Fitzgerald; 1 μ g/mL).

Statistical Analysis

For statistical purposes all variables for each experimental condition were averaged across the cardiomyocytes from each heart to provide a single value per heart. All data are expressed as mean $\pm$ SEM. Student's t-test or one-way ANOVA with a Holm-Sidak post-hoc correction were used as appropriate.

Results

EPO Effects on Intact Murine Papillary Muscle

Twitch dynamics were determined in isolated murine left ventricular papillary muscles at 37°C. For the studies in mouse myocardium, six muscle preparations were obtained from separate hearts. Figure 1A shows a representative tracing of a muscle twitch at baseline and 5 minutes after the addition of EPO. Diastolic tension in the papillary muscle strips was unchanged over the time course of the experiments (1.4 ± 0.6 mN/mm² at baseline and 1.3 ± 0.8 mN/mm² at 20 minutes of EPO administration; $P = \text{NS}$). Peak twitch force generated by the papillary muscles increased from 3.1 ± 0.4 mN/mm² at baseline to 4.2 ± 0.5 mN/mm² after 5 minutes, representing a 35% increase. Force was also increased at the 10, 15 and 20 minute time-points (Figure 1B; $P < 0.05$). After 5 minutes of EPO treatment peak rate of force development (+ dF/dT) was increased by 49%, from the baseline value of 99.8 ± 26.9

mN/mm²s to 148.8±32.9 mN/mm²s ($P<0.001$). Peak relaxation rate ($-dF/dT$) was increased by 52% after 5 minutes of EPO treatment, from -75.8 ± 17.8 mN/mm²s to -115.2 ± 24.2 mN/mm²s ($P<0.01$). Treatment with EPO resulted in a modest change in the relative relaxation time to 50% (7% reduction after 5 minutes, $P<0.05$) with no significant change in the relaxation time to 90% of peak force.

Effects of EPO on Human Left Ventricular Strip Preparations

We analyzed the effects of 50 U/mL of EPO on developed force in excitable left ventricular muscle strips obtained from five patients undergoing coronary artery bypass surgery ($n = 3$) or aortic valve replacement ($n = 2$). All patients had a normal ejection fraction. EPO increased developed force generation in all strip preparations. Compared to baseline, EPO increased twitch force at 10 minutes by an average of 62% ($P=0.025$; Figure 1C) with no change in diastolic tension over baseline.

Positive Inotropic and Lusitropic Effects of EPO on Isolated Murine Cardiomyocytes

To determine if the positive contractile effects of EPO in intact muscle strips are also evident at the single cell level, isolated, unloaded cardiomyocyte experiments were performed. Representative recordings obtained from control cardiomyocytes and cardiomyocytes exposed to 50 U/ml of EPO for 90 min are shown in Figure 2A. Table 1 summarizes the overall analysis of cardiomyocytes obtained from 7 mice. EPO increased cardiomyocyte contractility, as demonstrated by a 41% increase in peak sarcomere shortening ($P=0.012$) and a 45% increase maximum shortening velocity ($P=0.004$). In addition, EPO treated cardiomyocytes demonstrated a 54% increase in maximum relengthening velocity ($P=0.013$).

These findings confirm that the positive inotropic and lusitropic effects of EPO observed in excitable papillary muscle preparations are also present at the level of the single cardiomyocyte. The slower onset of action of EPO in isolated myocytes compared to papillary muscle preparations may be due to the fact that the muscle strip experiments were performed at 37°C whereas EPO incubation in the isolated myocytes was performed at room temperature.

Intracellular Ca²⁺ Dynamics

To determine if the positive inotropic and lusitropic effects of EPO are mediated through enhanced intracellular calcium dynamics, Ca²⁺ transients were measured in EPO treated cardiomyocytes in comparison to controls. Representative signal averaged intracellular Ca²⁺ transients from a control cardiomyocyte and a cardiomyocyte exposed to 50 U/ml of EPO are shown in Figure 2B. The results from cardiomyocytes obtained from 9 different animals are summarized in Table 2. Calcium transient characteristics in the EPO treated cardiomyocytes in comparison to controls demonstrated an 18% increase in the peak systolic Ca²⁺ signal ($P=0.006$), and a 7% decrease in time to peak Ca²⁺ ($P=0.019$).

Dose Dependence and Time Course of the Contractile Response to EPO

The results of EPO dose response and time course experiments are shown in Figure 3. The effects of 0, 0.1, 1, 10 and 50 U/ml on maximal sarcomere shortening were determined from cardiomyocytes isolated from 6 hearts (Figure 3A). Sarcomere shortening was significantly increased at an EPO concentration of 10 U/ml ($P<0.05$), with no further change at the higher concentration of 50 U/ml. The time course of the EPO contractile effect was studied in cardiomyocytes obtained from 4 mice (Figure 3B). Sarcomere shortening was significantly enhanced and maintained over baseline after 90 minutes of incubation with 50 U/ml EPO at room temperature ($P<0.05$).

Selective Heterodimeric EPO Receptor Stimulation

Treatment of mouse cardiocytes with compounds that selectively activate the heterodimeric EPO receptor resulted in positive cellular inotropy and lusitropy that was indistinguishable from EPO treatment (Figure 4; Table 3). Specifically, treatment with CEPO (0.5mg/ml) or ARA290 (16nmol/L) resulted in an approximate doubling of peak shortening and relengthening velocities over control. Percent sarcomere shortening was also significantly increased. Experiments using a sequenced scrambled peptide had no effect on contractility (data not shown). These data indicate that the effects of EPO on cardiac contractility can be mediated through the non-erythropoietic, heterodimeric EPO receptor.

EPO's Effects on Skinned Strips

To further investigate the direct effect of EPO on myofilament function, we treated isolated myocardial strips with EPO (50 U/mL) for 90 minutes prior to chemical skinning. No difference in the pCa-tension relation was observed between EPO treated and untreated control strips (Figure 5A; Table 4). In addition, no difference was observed after phosphatase treatment of EPO treated strips. By inducing small sinusoidal length changes in the skinned strips, elastic and viscous stiffness were measured as a function of the frequency of perturbation in EPO treated (50 U/ml) and control strips. At maximum calcium activation, there was no difference in the elastic modulus between EPO treated and control strips (Figure 5B). In contrast, the viscous modulus, i.e., the out-of-phase response, was significantly increased with EPO treatment over the frequency range of 12–35 Hz ($P<0.05$; Figure 5C). These differences were abrogated with phosphatase treatment (data not shown) indicating that the change in myofilament function produced by EPO is mediated by myofilament phosphorylation.

Oscillatory work production (force $\times$ length) was assessed in calcium activated myofilaments (pCa 4.8). The change in tension (stress) of the myofilaments in response to length perturbation (strain) results in a frequency-dependent transfer of mechanical work between the length motor and the myofilaments. Perturbation at 3 Hz (solid line) gave rise to a stress that lagged behind the strain (dotted line) while at 12 Hz (dashed line) the stress led the strain (Figure 6A). These stress-strain relationships are analogous to ventricular pressure-volume relationships where counter-clockwise loops reflect work produced by the muscle. Thus, at 3 Hz, the counter-clockwise stress-strain relationship reflects mechanical work produced by the myofilaments and transferred to the motor (Figure 6B). In contrast, at 12 Hz mechanical work is absorbed by the myofilaments. Incubation in EPO prior to chemical-skinning of myocardial strips led to a significant increase ($P<0.01$) in oscillatory work produced by the myofilaments (Figure 6C, Table 4), i.e., higher work output for frequencies below ~ 5 Hz, i.e., within the physiologic range. This pattern was reversed at higher frequencies. Importantly, phosphatase treatment reversed the effects of EPO on oscillatory work production. Such a shift in the relationship between frequency and myofilament oscillatory work may contribute to the enhanced peak shortening observed at lower frequencies in myocytes exposed to EPO (Figure 6D).

EPO Mediated Intracellular Signaling

To delineate the intracellular signaling pathways responsible for the EPO effect on contractility, isolated cardiomyocytes were divided into three aliquots. One aliquot served as control and one aliquot was incubated with 50 U/ml EPO for 90 minutes, as previously described. The third aliquot was incubated for 90 minutes with both 50 U/ml EPO and one of the specific intracellular signaling pathway inhibitors. For each inhibitor, cardiomyocytes from at least four hearts were analyzed. The concentration of the inhibitors was chosen according to values previously demonstrated to provide effective blockade [12,14]. It has been previously shown in isolated cardiomyocytes that wortmannin, and chelerythrine do

not affect cardiomyocyte contractility under basal conditions [15,16]. Cardiomyocytes exclusively treated with EPO showed an increase in sarcomere shortening when compared to controls ($78 \pm 19\%$, $P < 0.001$). When phosphatidylinositol-3-kinase (PI3-K) activity was blocked with the addition of 100 nmol/L wortmannin, sarcomere shortening increased by only $18 \pm 13\%$ (Figure 7; $P = \text{NS}$), demonstrating a marked dependence of the EPO mediated contractile effect on this pathway. The involvement of protein kinase C (PKC) in the EPO elicited contractile effect was demonstrated with addition of the non-isozyme specific PKC inhibitor, chelerythrine, at a concentration of 5 $\mu\text{mol/L}$. Chelerythrine markedly inhibited the inotropic effect of EPO, with an increase in sarcomere shortening of only $7 \pm 16\%$ ($P = \text{NS}$). These results indicate that PKC is the likely down-stream effector of PI3-K. To clarify if classical (Ca^{2+} dependent) or novel (Ca^{2+} independent) PKC isoforms are involved we conducted experiments with Gö6976, a PKC inhibitor that specifically inhibits the Ca^{2+} dependent PKC isoforms α and β [14]. Gö6976 treatment (100nmol/L) alone elicited a non-significant increase in sarcomere shortening of $18 \pm 11\%$ ($P = 0.202$, Supplement Table 1). Given that PKC α and β both have been shown to depress myocyte contractility [17,18], the borderline increase in contractility observed with Gö6976 may represent inhibition of basal PKC α and β signaling. When compared with EPO treated cardiomyocytes the addition of Gö6976 did not block the EPO mediated effect (Figure 7, Supplement Table 1), indicating that the classical PKC isoforms are not the primary mediators of the EPO contractile effect.

PKC ϵ Activation With EPO

Intracellular activation of PKC δ and ϵ was determined by assessing relative abundance of these kinases in the cytosolic and particulate fraction of EPO treated fibers in comparison to controls. Cellular fractionation of myocardial strips with and without treatment of EPO demonstrated a significant increased in PKC ϵ abundance ($P = 0.002$) in the particulate fraction of the EPO treated myocardial strips when compared to untreated samples (Figure 8). No change in the cellular distribution of PKC δ was observed with EPO treatment. An increase in PKC ϵ in the particulate fraction indicates activation of this isoform with EPO treatment. Collectively, these data strongly suggest that PKC ϵ mediates the modulation in cardiomyocyte contractility by EPO.

Discussion

In these experiments employing left ventricular excitable muscle preparations and single cardiomyocytes, we demonstrate that EPO markedly affects cardiac contractile dynamics. A substantial positive inotropic effect was observed, manifested by an increase in peak developed force in excitable muscle strips as well as an increase in the rate and amplitude of systolic sarcomere shortening in isolated cardiomyocytes. These contractile effects are associated with an increase in the rate and magnitude of systolic calcium release, as well as a direct, calcium-independent myofilament effect. Mechanical studies in skinned muscle fibers demonstrate an increase in oscillatory work production with EPO treatment at lower oscillatory frequencies. Moreover, EPO produced positive lusitropic effects in both excitable strips and isolated myocytes. Given the absence of changes in calcium cycling or myofilament mechanical parameters that would account for the enhanced rate of relaxation, it is likely that the lusitropic effects of EPO are simply due to an increase in the recoil force of the muscles' elastic elements, e.g., titin, that result from the increased extent of sarcomere shortening.

These direct effects of EPO observed in single murine cardiomyocytes and excitable muscle preparations may account for some of the results reported in previous studies. Improved left ventricular function has been observed after EPO treatment in Langendorff perfused rat hearts with or without ischemia-reperfusion [12,19,20] and an *in situ* post-myocardial infarction model in the rabbit [21]. Both systolic and diastolic *in vivo* hemodynamic

parameters were significantly improved in rats treated with darbepoetin after myocardial infarction [1]. Finally, in a small clinical trial, a significant increase (5.5 %) in ejection fraction was observed in EPO treated heart failure patients with anemia [4]. Due to the cytoprotective, vascular, and hematopoietic effects of EPO, one cannot deduce a direct contractile effect of EPO or darbepoetin from the above studies.

Serum levels in the treatment arms of clinical trials demonstrating a beneficial effect of EPO in stroke [22] and myocardial infarction patients [23] have been in the range of 4–6 U/ml. Our results show that the positive contractile effect of EPO is seen at concentrations as low as 10 U/ml, which is close to the clinical dosing range.

EPO is known to activate several intracellular signaling pathways. When EPO binds to its receptor, the receptor dimerizes, which in turn activates Janus kinase 2 and subsequently triggers the activation of the PI3-K/Akt and STAT 5 pathways [24]. We show that the observed contractile effects of EPO are dependent on the PI3-K pathway, which has been previously demonstrated to mediate the anti-apoptotic effects of EPO [25] as well as its cytoprotective effects in ischemia-reperfusion injury [3]. Moreover, PKC, a downstream effector of PI3-K, has been shown to be pivotal in mediating the cytoprotective effects of EPO, as co-administration of EPO with the non-isozyme specific PKC inhibitor chelerythrine abolished the beneficial effect of EPO in ischemia reperfusion models [12,26]. PKCs α , β , δ and ϵ are the only known PKC isozymes expressed in the adult mouse heart [27]. Our results using the PKC α and β selective blocker Go6976 demonstrate that the observed effects are independent of PKC α and β . Finally, cellular fractionation demonstrated an increase in PKC ϵ , but not PKC δ in the particular fraction with EPO treatment, indicating that PKC ϵ is likely directly involved in mediating the effects of EPO on sarcomere dynamics.

Over expression of PKC ϵ in adult rat cardiomyocytes results in enhanced contractility [28]. PKC ϵ translocates from the nucleus and peri-nucleus to cross-striated structures [29], bringing the activated isoform in close proximity to the sarcomeric proteins. Furthermore, EPO induced translocation of PKC ϵ but not PKC δ has been previously demonstrated [12]. The fact that the EPO effect on cardiac contractility is delayed in onset also supports the involvement of a complex intracellular signaling process such as the translocation events necessary to activate PKC isozymes. Previous in vitro experiments have shown that PKC mediated effects on cardiac contractility take at least several minutes to occur [28] and that PKC ϵ translocation may take up to two hours to achieve maximal activation [30]. We also demonstrated that the effect of EPO on myofilament contractile function is reversed with phosphatase treatment. These data are consistent with EPO ultimately inducing PKC ϵ mediated phosphorylation of contractile proteins.

A frequently cited limitation of EPO therapy is the potential associated increase in red blood cell mass, thrombogenicity and blood pressure, which could be detrimental in patients with heart failure. Here we demonstrate positive inotropic and lusitropic effects using compounds that selectively activate the non-erythropoietic, heterodimeric EPO receptor [5]. In contrast to EPO, CEPO (which selectively stimulates the heterodimeric EPO receptor) has no apparent adverse hemodynamic or thrombogenic effects, and enhance renal blood flow and sodium excretion in a preclinical rat model [31]. Thus the use of these novel compounds in patients may offer the possibility of augmented cardiac performance without adverse hematologic or hemodynamic consequences.

In summary, despite numerous advances in treatment, the diagnosis of heart failure still is associated with high 5 year mortality and rehospitalization rates. Our findings reveal substantial positive inotropic and lusitropic effects of EPO in both isolated cardiomyocytes

and in excitable left ventricular muscle preparations which appear to be mediated by activation of PI3-K and PKC ϵ . Further studies are needed to determine if the effects seen in isolated cell and tissue preparations translate to sustainable, positive contractile effects in the intact heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Richard Lachapelle, Zengyi Chen, Yuan Wang and Stephen Bell assisted in performing experiments. We would like to thank Michael Brines and Anthony Cerami of Warren Pharmaceuticals.

Funding Sources

Work was supported by NIH grants 5R01HL077637, R01HL086902 and 5T32HL007647-17.

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Highlights

- Myocardial contractile function is enhanced with erythropoietin (EPO) treatment.
- Heterodimeric EPO receptor stimulation yields similar contractile effects.
- The inotropic effect of EPO is mediated via novel PKC isoform signaling.
- EPO enhances the contractility of both mouse and human myocardium.

Figure 1. Effect of EPO on left ventricular papillary muscle strips. A) Representative tracing of a muscle twitch at baseline and 5 minutes after the addition of 50 U/ml to the superfusion solution. B) Relative effect of EPO on peak force generation (papillary muscles from 6 hearts). C) Twitch response human ventricular myocardium at baseline and after 10 minutes of 50 U/ml EPO exposure (n=5). * $P < 0.05$ versus baseline.

Figure 2.

Representative sarcomere shortening and fura-2 fluorescence ratio transients at 8 Hz and 37°C without and with EPO incubation at 50 U/mL for 90 min. A) Sarcomere shortening was markedly enhanced by EPO exposure (myocytes from 7 hearts; see Table 1). B) Fluorescence ratio transient was significantly affected by EPO exposure (myocytes from 9 hearts; see Table 2).

Figure 3.

Dose and time responses to EPO at 8 Hz and 37° C. A) Peak sarcomere shortening of multiple cardiomyocytes at each EPO concentration was averaged to produce a response for each heart. Number of hearts is 6. The dose response to EPO revealed a maximum change in peak shortening relative to no EPO at 10 and 50 U/mL. B) Peak sarcomere shortening was monitored over 120 min during continuous pacing at 8 Hz. The average of multiple cardiomyocytes from 4 hearts indicated that a maximum response to 50 U/mL EPO occurred after 90 min exposure. * $P < 0.05$ versus control.

Figure 4.

Peak sarcomere shortening for EPO, CEPO, or ARA290 treated cells in comparison to control. Similar to EPO, CEPO and ARA290 enhanced sarcomere shortening (myocytes from 5–7 hearts; see Table 3). * $P < 0.05$ versus control.

Figure 5.

EPO effects on myofilament function. A) Normalized tension-pCa relationships demonstrating no change in the myofilament calcium sensitivity with exposure to EPO or additional phosphatase. B) The elastic modulus, i.e., the in-phase response of the tension in response to length perturbations, was not significantly affected by EPO. C) The viscous modulus, i.e., out-of-phase response, was significantly increased with EPO incubation over the frequency range of 12–35 Hz ($P < 0.05$). No EPO = control. EPO = exposure to 50 U/mL for 90 min. EPO + Ph = additional exposure to 6 U/mL alkaline phosphatase for 10 min immediately before experiment. Strips were obtained from 4 hearts.

Figure 6.

Oscillatory work production by calcium activated myofilaments. A) The change in tension (stress) of the myofilaments to length perturbation (strain) results in a frequency-dependent the transfer of mechanical work between the length motor and the myofilaments. Perturbation at 3 Hz (solid line) gave rise to a stress that lagged behind the strain (dotted line) while at 12 Hz (dashed line) gave rise to a stress that led the strain. B) These stress-strain relationships are analogous to pressure-volume relationships where counter-clockwise loops reflect work produced by the muscle. At 3 Hz, the stress-strain relationship resulted in mechanical work produced by the myofilaments. This work produced in the myofilaments is inherently independent of calcium cycling. At 12 Hz, mechanical work is absorbed by the myofilaments. C) Incubation in EPO prior to chemical-skinning of myocardial strip led to a significant ($P < 0.05$) increase in oscillatory work production by the myofilaments and which is reversed with phosphatase treatment. D) The frequency shift in myofilament oscillatory work may have contributed to the enhanced peak shortening at lower frequencies in myocytes exposed to EPO.

Figure 7.

EPO effects on sarcomere shortening in the presence of wortmannin, chelerythrine and Gö6976. Data is presented as mean percent change of sarcomere shortening when compared to control. N=27–40 cardiomyocytes for each group from four to nine different animals, * $P < 0.05$ versus control, wortmannin and chelerythrine treated cardiomyocytes. See Supplement Table 1.

Figure 8. Relative abundance of PKC ϵ and PKC δ isoforms with EPO exposure (50 U/ml for 90 min) versus control samples in cytosolic and particulate cellular fraction. Values reported were normalized using associated GAPDH blots. Data is from 12 EPO-treated and 12 control myocardial strips. * $P < 0.05$ versus control.

Table 1

Characteristics of sarcomere contraction transients at 8 Hz.

	Control (n=7)	EPO (n=7)	paired t-test
SL _{dias} (μm)	1.669±0.029	1.672±0.026	0.955
Max Shortening Velocity (μm/s)	-3.09±0.24	-4.47±0.32	0.004 *
Peak Shortening (% of SL _{dias})	3.21±0.26	4.54±0.37	0.012 *
Time to Peak (ms)	48.0±3.8	47.5±3.0	0.925
Max Relengthening Velocity (μm/s)	2.41±0.30	3.70±0.32	0.013 *
Time to 50% return to SL _{dias} (ms)	63.9±6.5	64.3±5.3	0.964
Time to 75% return to SL _{dias} (ms)	71.5±6.2	72.7±4.7	0.883
Time to 90% return to SL _{dias} (ms)	88.4±4.1	83.8±1.7	0.319

Results from multiple (≥5) cardiomyocytes from each heart were averaged to provide a single value for each heart. SL = sarcomere length. n = number of hearts.

* denotes statistical significance.

Table 2

Characteristics of calcium transients at 8 Hz.

	Control (n=9)	EPO (n=9)	paired t-test
R_{dias}	0.457±0.055	0.557±0.086	0.072
dR/dt_{max} (s^{-1})	32.98±2.04	37.80±3.52	0.14
Time to dR/dt_{max} (ms)	6.2±0.40	4.9±0.2	0.026 *
$R_{\text{peak}} - R_{\text{dias}}$	0.306±0.016	0.362±0.022	0.006 *
Time to R_{peak} (ms)	17.9±0.6	16.7±0.4	0.019 *
$-dR/dt_{\text{max}}$ (s^{-1})	-6.16±0.44	-7.13±0.94	0.586
Time to $-dR/dt_{\text{max}}$ (ms)	37.2±2.2	35.5±2.4	0.583
Time to 50% return to R_{dias} (ms)	51.7±1.7	51.7±1.6	0.988
Time to 90% return to R_{dias} (ms)	85.9±2.0	86.9±0.8	0.685

Results from multiple (≥ 5) cardiomyocytes from each heart were averaged to provide a single value for each heart. R = fluorescence ratio of Fura-2 excited at 365 nm (numerator) and 410 nm (denominator). n = number of hearts.

* denotes statistical significance.

Table 3

Characteristics of sarcomere contraction transients at 8 Hz.

	Control (n=6)	EPO (n=7)	CEPO (n=5)	ARA290 (n=5)
SL _{dias} (μm)	1.61±0.02	1.65±0.02	1.63±0.01	1.64±0.02
Max Shortening Velocity (μm/s)	-2.35±0.31	-4.02±0.53 *	-4.36±0.17 *	-3.96±0.28 *
Peak Shortening (% of SL _{dias})	2.85±0.17	4.50±0.42 *	4.92±0.19 *	4.38±0.21 *
Max Relengthening Velocity (μm/s)	1.50±0.23	3.51±0.50 *	3.53±0.20 *	3.20±0.21 *
Time to 90% return to SL _{dias} (ms)	86.6±4.4	90.1±3.1	90.3±2.7	89.0±2.0

Results from multiple (≥5) cardiomyocytes from each heart were averaged to provide a single value for each heart. SL = sarcomere length. n = number of hearts.

* $P < 0.05$ versus control.

Table 4

Myofilament sensitivity to calcium activation was not affected by EPO or additional phosphatase treatment.

	No EPO (n=4)	EPO (n=4)	EPO+Ph (n=4)
$T_{\min}$ (mN.mm ⁻²)	3.6±0.4	4.4±0.7	3.5±0.4
T_{dev} (mN.mm ⁻²)	21.4±1.5	20.0±0.8	22.9±2.0
pCa ₅₀	5.80±0.02	5.78±0.05	5.76±0.05
n _{Hill}	3.48±0.57	3.43±0.16	3.60±0.26
F _{Wosc} (Hz)	5.3±0.6	3.4±0.1*	6.4±0.9

The frequency of maximum oscillatory work (F_{Wosc}) at maximum calcium activation was significantly reduced by EPO. No EPO = control incubation in 95% O₂-5% CO₂ bubbled Krebs solution for 90 min at room temperature (RT). EPO = exposure to 50 U/mL. EPO+Ph = EPO exposure plus treatment with 6 U/mL alkaline phosphatase. n = number of strips.

* $P < 0.01$ versus No EPO.