Acute hemodynamic effects of erythropoietin do not mediate its cardioprotective properties

Ismayil Ahmet, Edward G. Lakatta and Mark I. Talan*

Laboratory of Cardiovascular Sciences, National Institute on Aging, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD 21224, USA

*Author for correspondence (talanm@grc.nia.nih.gov)

Biology Open 1, 1049–1053
doi: 10.1242/bio.20122378
Received 25th June 2012
Accepted 31st July 2012

Summary

Activation of nitric oxide (NO) signaling is considered, at list partially, a mechanistic basis for EPO-induced cardioprotection. Surprisingly, hemodynamic response subsequent to NO activation after EPO administration has never been reported. The objectives of this study were to evaluate the acute hemodynamic and cardiovascular responses to EPO administration, to confirm their NO genesis, and to test the hypothesis that EPO-induced cardioprotection is mediated through cardiovascular changes related to NO activation. In *Experiment 1*, after 3000 U/kg of rhEPO was administered intravenously to Wistar rats, arterial blood pressure, monitored via indwelling catheter, progressively declined almost immediately until it leveled off 90 minutes after injection at 20% below control level. In *Experiment 2* the 25% reduction of mean blood pressure, compared to control group, was observed 2 hours after intravenous injection of either 3000 or 150 U/kg of rhEPO. Detailed pressure–volume loop analyses of cardiac performance (*Experiment 3*) 2 hours after intravenous injection of human or rat recombinant EPO (3000 U/kg) revealed a significant reduction of systolic function (PRSW was 33% less than control). Reduction of arterial blood pressure and systolic cardiac function in response to rhEPO were blocked in rats pretreated with a non-selective inhibitor of nitric oxide synthase (L-NAME). In *Experiment 4*, 24 hours after a permanent ligation of a coronary artery, myocardial infarction (MI) measured 26±3.5% of left ventricle in untreated rats. MI in rats treated with 3000 U/kg of rhEPO immediately after coronary ligation was 56% smaller. Pretreatment with L-NAME did not attenuate the beneficial effect of rhEPO on MI size, while MI size in rats treated with L-NAME alone did not differ from control. Therefore, a single injection of rhEPO resulted in a significant, NO-mediated reduction of systemic blood pressure and corresponding reduction of cardiac systolic function. However, EPO-induced protection of myocardium from ischemic damage is not associated with NO activation or NO-mediated hemodynamic responses.

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Key words: Erythropoietin, Nitric oxide, Arterial blood pressure, Cardiac function, Myocardial infarction, Cardioprotection

Introduction

Erythropoietin (EPO) is a glycoprotein hormone whose primary role is stimulation and maintenance of red cell production via inhibition of apoptosis in blast cells, thus regulating the balance between erythrocyte production and loss (Sawada et al., 1990; Wickrema et al., 1992; Silva et al., 1996; Tilbrook and Klinken, 1999). The clinical potential of EPO has long been recognized, and recombinant human EPO (rhEPO) is widely used in the treatment of anemia (Fisher, 1997; Fisher, 2003). During the last decade new properties of EPO, other than erythropoietic, mainly cytoprotective, have been discovered and extensively researched (Bogoyevitch, 2004; Lipšic et al., 2006; Koul et al., 2007; Riksen et al., 2008; Latini et al., 2008; Minnerup et al., 2009; Vogiatzi et al., 2010; Jerndal et al., 2010; Rathod and Salahudeen, 2011), promising new therapeutic approaches in areas range from nervous tissue damage to myocardial infarction to chronic heart failure. Tissue protective properties of EPO are associated with ERK and PI3K signaling pathways (Bullard et al., 2005), resulting in elevation of mitochondrial permeability threshold to ROS, and thus with increased mitochondrial tolerance to ischemic damage (Moon et al., 2006). While erythropoietic properties of EPO are undoubtedly associated with stimulation of erythropoietin receptors (EPO-R), the tissue protective effect seems to result from its binding to a hetero-dimer formed by EPO-R and common β receptor (cβR) (Brines et al., 2004; Brines, 2010).

Nitric oxide (NO) is one of signaling pathways associated with EPO (Bahlmann et al., 2004; Sautina et al., 2010). It has been reported that rhEPO stimulates vascular NO production directly (Banerjee et al., 2000; Kanagy et al., 2003; Beleslin-Cokic et al., 2004) or indirectly through increased shear stress in endothelial cells (Walker et al., 2000). Some reports attribute the rhEPO-induced cardioprotection to NO activation (Bullard and Yellon, 2005; Teng et al., 2011).

The main side effect and limiting factor of chronic rhEPO therapy is a systemic hypertension (Vaziri, 1999). The mechanism of rhEPO-induced hypertension has been understood as a result of increased blood viscosity and reduced hypoxic vasodilation in rhEPO-treated anemic patients (Nonnast-Daniel et al., 1988; Schaefer et al., 1988). A possibility of direct vasopressor effect

of EPO (Samtleben et al., 1988) or indirect effect through activation of renin–angiotensin system in tissues (Eggena et al., 1991) was also implied. Thus, NO activation in rhEPO patients was considered to be a factor limiting the hypertensive response through compensatory vasodilation (Kanagy et al., 2003).

Based on the consistent experimental evidence of rhEPO-induced NO activation, we hypothesized that a single bolus injection of rhEPO would produce an acute hemodynamic response associated with vasodilatation that, by itself, could be a mechanistic factor for an EPO-induced protection of myocardium from ischemic damage.

Results

The results of *Experiment 1* are presented in Fig. 1. Arterial blood pressure began to fall almost immediately after an intravenous bolus injection of 3000 U/kg of rhEPO (Fig. 1A). Both systolic and diastolic pressures continued to fall until leveling off 90 minutes following the EPO injection (Fig. 1B,C); the 30 min averages of both systolic and diastolic blood pressure were consistently and significantly lower in rhEPO treated group compared to control.

Table 1 presents the results of *Experiment 2*, in which blood pressure was measured 2 hrs after high (3000 U/kg, cardioprotective) or low (150 U/kg, usual therapeutic) doses of rhEPO were injected intravenously. Two hours following injections of either dose of rhEPO the blood pressure in rhEPO-treated rats was significantly ($p<0.05$) lower than in control rats.

The results of *Experiment 3*, acute effect of EPO on hemodynamic and cardiovascular function, are presented in Table 2. Bolus injections of 3000 U/kg of rhEPO or an equal dose of recombinant rat EPO (rrEPO) were administered intravenously immediately after pretreatment either with L-NAME (15 mg/kg) or with physiological saline (vehicle). Two hours after injection rats were anesthetized and their hemodynamic and cardiovascular functions were assessed via pressure–volume loop analyses. Systolic, diastolic and mean arterial pressures were significantly lower in rats treated with either human or rat recombinant EPO compared to the control group (columns 2 or 3 *vs* 1). All parameters reflecting cardiovascular performance were similar in rats treated with either rhEPO or rrEPO. Indices reflecting cardiac systolic function (ESP, dP/dt+, and PRSW) were significantly lower in EPO treated rats compared with control (in rrEPO treated animals, however, reduction in PRSW did not reach statistical significance). Pretreatment with L-NAME (columns 4, 5, and 6) completely blocked the effects of EPO on blood pressure and cardiovascular performance.

The results of *Experiment 4* are presented in Fig. 2. In the control, untreated, group 24 hrs after coronary ligation the area of myocardium at risk (AAR) covered approximately 50% of the LV. AAR was similar among all groups. The resulting MI in untreated (control) animals occupied 50% of AAR or 25% of LV. Administration of rhEPO immediately after coronary ligation resulted in 60% smaller MI than in control, not treated with rhEPO rats. Treatment with L-NAME without subsequent EPO administration resulted in the MI size similar to that of control, untreated animals. Pretreatment with L-NAME did not affect the reduction of MI size produced by rhEPO treatment.

Discussion

There is a disagreement whether chronic treatment with EPO is associated with activation of NO, which serves as a limiting factor to EPO-related hypertension (Kanagy et al., 2003) or, in

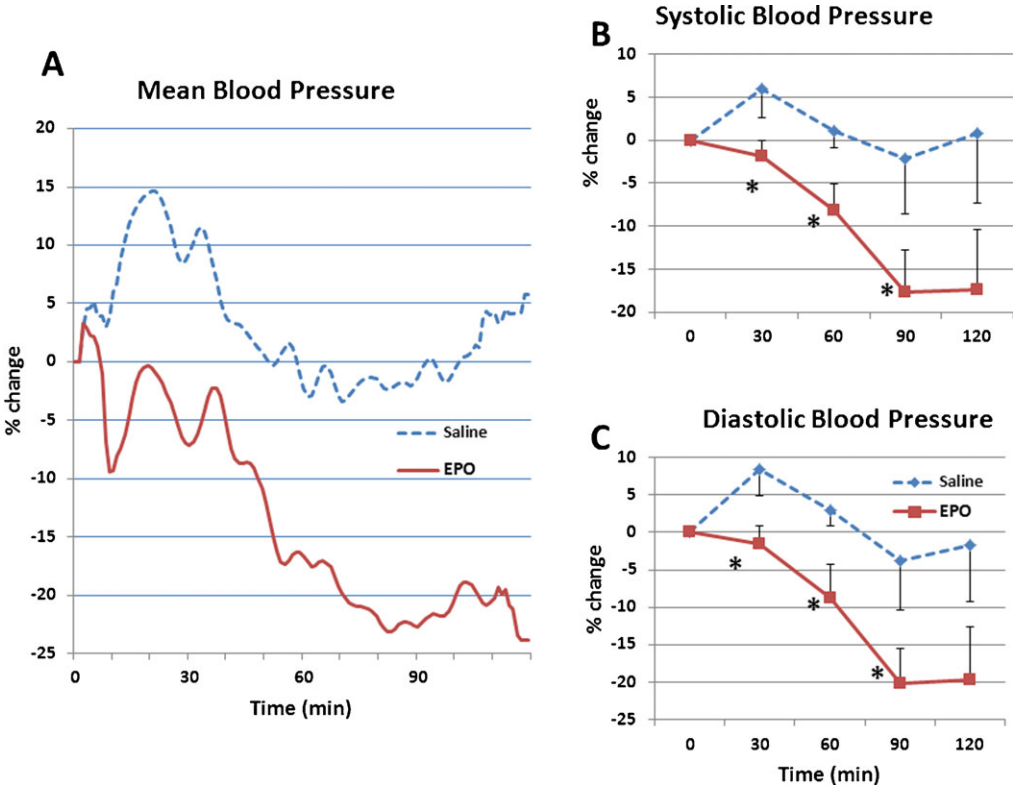

Fig. 1. Arterial blood pressure in rats during 2 hrs following intravenous bolus injection of 3000 U/kg of rhEPO. (**A**) Continuous recording of the mean blood pressure; (**B**) 30 min averages of systolic blood pressure; (**C**) 30 min averages of diastolic blood pressure.

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Table 1. Arterial blood pressure 2 hrs after intravenous bolus injection of 2 different doses of rhEPO (Means ± SEM).

Arterial BP	Control (n=11)	3000 U/kg (n=7)	150 U/kg (n=4)	ANOVA
Systolic (mmHg)	90±6	70±3#	72±3#	*
Diastolic (mmHg)	55±5	38±3#	39±2#	*
Mean (mmHg)	66±6	49±3#	50±2#	*

*p<0.05
#p<0.05 *vs* control, *post hoc* comparison

the opposite view, chronic application of EPO results in depression of NO synthase and actually may cause hypertension (Wang and Vaziri, 1999). Nevertheless, a predominant opinion is that a single EPO application is associated with activation of NO signaling. However, to the best of our knowledge our experiments are the very first to demonstrate that an acute hemodynamic response to a bolus injection of rhEPO is a significant and immediate reduction of arterial blood pressure. The very short latent period of reaction suggests that the acute hemodynamic response was, most probably resulting from triggering the fastest vasodilating signaling pathway – nitric oxide. This suggestion was confirmed by pretreatment with L-NAME, which completely blocked the acute hemodynamic response. Moreover, the hypotensive effect of rhEPO was similar in doses as high as 3000 U/kg (used is animal experiments on cardioprotection) and in doses as low as 150 U/kg (normal therapeutic dose for treatment of anemia). We have no reasonable explanation why this acute hemodynamic response to rhEPO was not observed previously. Moreover, our observation directly contradicts the prevailing view and even some direct experiments demonstrating that rhEPO in the dose as high as 1000 U/kg had no acute hemodynamic effect in normotensive rats and elicited hypertensive response in spontaneously hypertensive rats (Tojo et al., 1996); however, the blood pressure in that experiments was recorded only during the first 5–10 minutes after rhEPO injection. Resolution of this contradiction should probably wait for independent confirmation of our findings. One thing clarified in the present study was that the observed effect was not due to a spurious property of recombinant human EPO when administered to a rat – administration of recombinant rat's EPO had exactly the same effect.

Another interesting revelation of the present study is a significant reduction of systolic heart function observed 2 hrs after rhEPO administration demonstrated via comprehensive pressure–volume loop analyses. The reduction of ESP, dP/dt max, and especially PRSW is consistent with a reduced arterial pressure, and indicates lower cardiac work and, therefore,

Table 2. Cardiovascular response 2 hours following a bolus injection of erythropoietin (Mean±SEM).

	Vehicle			L-NAME			ANOVA	
	Control (n=11)	rhEPO (n=7)	rrEPO (n=7)	Control (n=5)	rhEPO (n=5)	rrEPO (n=5)		
Column numbers	1	2	3	4	5	6	H	R
BW (g)	492±13	494±6	474±15	490±16	506±15	492±27		
HR (beats/min)	325±11	306±12	318±6	346±14	347±6	362±14		
ESV (μL)	222±24	247±44	253±37	228±36	222±24	235±47		
EDV(μL)	388±22	400±40	383±23	335±26	284±25	315±39		
ESP (mmHg)	85±4	**66±5**	**68±3**	108±13	114±4	125±2	*	*
EDP (mmHg)	4.0±0.4	4.7±0.5	4.1±0.6	2.4±0.1	2.4±0.3	2.5±0.4	*	*
SV (μL)	220±18	222±41	203±21	148±21	102±9	96±16	*	*
EF (%)	56±4	52±7	52±6	42±7	35±5	31±5		*
CO (mL/min)	72±7	68±12	66±7	49±5	35±3	35±6	*	*
Ea (mmHg/μL)	0.39±0.01	0.4±0.09	0.37±0.05	0.83±0.22	1.11±0.08	1.44±0.24	*	*
dP/dt+ (mmHg/sec)	7136±454	**5420±415**	**5275±228**	6143±644	6151±244	7046±313	*	*
dP/dt− (mmHg/sec)	7303±691	5458±811	**4783±362**	7890±1086	8425±495	10072±244		*
Tau (ms)	7.0±0.3	7.4±0.3	7.8±0.2	8.1±0.3	8.8±0.2	8.4±0.4	*	*
dP/dt-EDV (mmHg/sec/μL)	17.6±2.7	14.4±2.0	17.1±3	34.2±8.9	33.8±4.0	44.1±7.3	*	*
PRSW (mmHg*μL/μL)	51.8±4.2	**34.6±3**	44.8±7	62.2±8.0	73.7±9.4	80.3±4.4	*	*
Ees (mmHg/μL)	0.20±0.02	0.19±0.04	0.18±0.02	0.75±0.18	0.87±0.18	0.88±0.14	*	*
Eed (10^{-3}*mmHg/μL)	13.6±4.0	14.6±2.1	11.9±2.8	20.8±7.5	20.8±2.4	25.4±5.9		
Ea/Ees	2.0±0.2	2.1±0.3	2.1±0.3	1.2±0.2	1.4±0.2	1.7±0.3	*	
BPs (mmHg)	90±5	**70±3**	**70±2**	111±11	114±4	125±3	*	*
BPd (mmHg)	55±5	**38±4**	**39±3**	86±13	91±4	103±2	*	
BPm (mmHg)	66±5	**49±3**	**50±3**	94±12	99±4	110±2	*	*
PP (mmHg)	35±1	32±2	**31±2**	25±3	23±1	22±1	*	*
PVR (mmHg/μL/min)	1.02±0.16	0.86±0.15	0.81±0.08	2.11±0.49	2.90±0.19	3.63±0.67	*	*

**P*<0.05; one way ANOVA for four groups
H. Experiment involving a human EPO (rhEPO), columns 1, 2, 4, 5
R. Experiment involving a rat EPO (rrEPO), columns 1, 3, 4, 6
Values marked by underlined and bold font = p<0.05, *post hoc* comparison between control and EPO-treated groups with Bonferroni correction for 2 comparisons

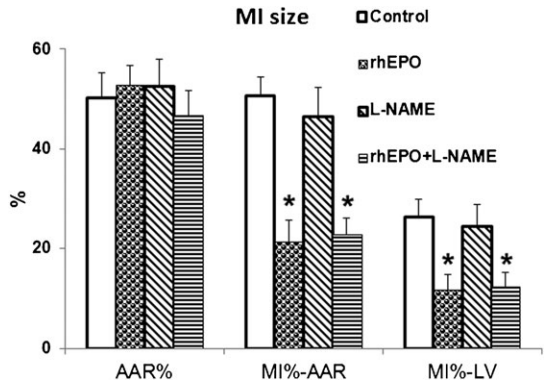

Fig. 2. AAR and MI size (as percent of AAR or LV) measured 24 hrs following a coronary ligation in untreated rats (control) and rats treated immediately after coronary ligation with rhEPO, L-NAME, or with L-NAME and rhEPO.

suggests lower oxygen requirement of the working myocardium. That fact alone can serve as a cardioprotective mechanism, and might be accountable for increase rhEPO-associated myocardial resistance to ischemic damage. This hypothesis, however, was not confirmed – while pretreatment with L-NAME prior to EPO administration completely blocked acute hemodynamic and related cardiac responses to rhEPO (Table 2), it did not affect the ability of EPO to reduce the MI size induced by a coronary ligation (Fig. 2).

The present finding that administration of L-NAME did not abrogate the effect of rhEPO to reduce the MI size following a coronary ligation indicates that cardioprotective properties of EPO are not mediated via NO signaling and contradicts conclusions of several previous reports (Bullard and Yellon, 2005; Teng et al., 2011; Burger et al., 2006; Joyeux-Faure et al., 2006). This discrepancy can be explained by several fundamental methodological differences between our study and those previous reports. Burger et al. reported that L-NAME blocked the protective effect of EPO in the culture of cardiomyocytes (Burger et al., 2006); Joyeux-Faure et al. used an ischemia/reperfusion paradigm in the isolated heart model and assessed the protective effect of EPO on the basis of heart contractility rather than measuring the size of resulting MI (Joyeux-Faure et al., 2006); Bullard and Yellon studied the cardioprotective effect of chronic EPO treatment – they treated rats 3 times per week for 3 weeks before conducting an acute ischemia/reperfusion experiment with and without L-NAME (Bullard and Yellon, 2005); and finally Teng et al. reported the lack of cardioprotection by EPO in $eNOS^{-/-}$ mice (Teng et al., 2011). Therefore, while methodological approaches used in previous reports allowed to suggest a possibility of NO involvement in EPO-induced cardioprotection, ours is the first study in which a possible involvement of NO in rhEPO-induced cardioportection was definitely excluded in the most straightforward model: the effect of rhEPO was assessed in genetically unmodified animals, cardioprotection was demonstrated by reduction of MI size 24 hrs following a coronary ligation, and L-NAME pretreatment and rhEPO were both administered immediately after coronary ligation.

While our experiments indicated that EPO-induced cardioprotection from ischemic damage is not mediated by NO activation, it had been recently reported that EPO-induced NO release requires the integration of EPO receptor and cβR with activation of Akt and PI3 kinase signaling (Su et al., 2011). Thus both non-erythropoietic effects of EPO – cardioprotection and acute hypotensive response – require involvement of cβR.

In summary, we are reporting a discovery of an acute, NO-mediated, hemodynamic effect of a bolus injection of rhEPO, i.e. a reduction in blood pressure and cardiac work. However, neither activation of NO signaling, nor NO-associated hemodynamic effects mediate cardioprotective properties of EPO. This newly discovered acute vasodilating effect of EPO *per se* will hardly have serious clinical implication, since chronic hypertensive effect of EPO is well documented. Nevertheless, a possible application of several recently reported non-erythropoietic derivatives of EPO should be clinically assessed.

Materials and Methods

Materials

Recombinant human erythropoietin, Procrit® (rhEPO) was purchased from Amgen. Recombinant rat erythropoietin (rrEPO) was purchased from R&D System (Minneapolis, MN).

Animals and experimental design

113 2–3-month-old male Wistar rats were purchased from Charles River and housed and studied in conformance with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals", manual 3040-2 (1999), with institutional Animal Care and Use Committee approval.

Experiment 1: Arterial blood pressure during the first 2 hrs after EPO administration

10 rats were anesthetized with Isoflurane (2% in Oxygen), intubated and artificially ventilated. Body temperature was maintained at 37℃ using heating pad and heat lamp. Left femoral artery and vein were isolated; a pressure catheter (Milar, 1F) was inserted into femoral artery and advanced proximally to the level of thoracic aorta. Arterial blood pressure was recorded continuously at 1 k/sec using a multi-channel recording system (PowerLab). After 10 min of baseline recording 3000 U/kg of rhEPO (n=6) or 1 ml of physiological saline (n=4) were injected as a bolus via right femoral vein. Blood pressure monitoring was continued for 2 hrs after drug administration.

Experiment 2: Arterial blood pressure after different doses of EPO administration

Under general anesthesia (2% of Isoflurane in Oxygen) rats were injected via femoral vein either with 3000 U/kg of rhEPO (n=7), or with 150 U/kg of rhEPO (n=5), or 1 ml/kg of physiological saline (n=11). Rats were allowed to recover from anesthesia. 2 hrs after injection rats were reanesthetized and 1F pressure catheter inserted via femoral artery into thoracic aorta (see above). Arterial blood pressure was recorded (see above) for 5 minutes.

Experiment 3: Arterial blood pressure and cardiovascular performance 2 hrs after EPO administration

Under general anesthesia 6 groups of rats were subjected to intravenous injections (see above) of 3000 U/kg of rhEPO (n=7), or 10 μg/kg of rrEPO, equivalent of 3000 U/kg (n=7), or 1 ml/kg of physiological saline (n=11). In 3 groups (n=5 in each) these injections were preceded with 15 mg/kg of a non-selective inhibitor of nitric oxide synthase (L-NAME). Rats were allowed to recover from anesthesia, but 2 hrs later were reanesthetized and hemodynamic and cardiovascular performances were analyzed via pressure–volume loops analyses (Ahmet et al., 2004). Briefly, after bilateral thoracotomy in the sixth intercostal space a 2-0 suture was placed around *vena cava inferior* for volume manipulation and a 1.4F-combined pressure–volume conductance catheter (Millar Instruments Inc., Houston, TX) was inserted into left ventricle (LV) through the apex. Prior to collecting cardiac data, the catheter was advanced to ascending thoracic aorta to record arterial blood pressure for 5 min. Than the catheter was repositioned into LV and measurements of cardiac function were commenced – pressure–volume loops were recorded and analyzed using PVAN analyzing software (v3.6; Millar Inc.). Traditional load-dependent hemodynamic indices, such as ejection fraction (EF), +dP/dt, −dP/dt, end-diastolic pressure (EDP), and isovolumic relaxation time constant (τ) were measured, and load-independent indices, i.e., end-systolic elastance (Ees), preload recruitable stroke work (PRSW), and end-diastolic stiffness (Eed) were determined or calculated. Arterial elastance (Ea) was calculated as index of vascular tension. Arterio–ventricular coupling (AV coupling), an index of cardiac work efficiency, was calculated as Ea/Ees.

Experiment 4: Myocardial infarction size 24 hrs after coronary ligation

Under general anesthesia (2% of Isoflurane in Oxygen) 40 rats were subjected to a permanent ligation of coronary artery. After opening the left side of the chest, the left anterior descending coronary artery was ligated by 7-0 surgical suture at the level of left atrial apex to induce myocardial infarction (MI), as previously described (Ahmet et al., 2005). Immediately after coronary ligation (<5 min), all animals received a single i.p. injection of either 3000 U/kg of rhEPO at (n=11), 1 cc/kg of physiological saline (n=11), 15 mg/kg of L-NAME (n=8) or both rhEPO and L-NAME (n=10). Chest was surgically closed, the residual air was evacuated through a needle puncture, and rats were weaned from anesthesia. 24 hrs after surgery, rats were anesthetized by Isolflurane, chests were opened bilaterally, and 2 ml of 5% Evans blue (Sigma) was injected rapidly into the left ventricle (LV) via apex, while the aorta was tightly closed by forceps to distinguish the perfused from underperfused areas. The hearts were removed, rapidly rinsed in phosphate-buffered cold saline, and atriums and great vessels were dissected from the ventricles. The ventricle was cut into 4 pieces along the short axis and kept in 4% triphenyltetrazolium chloride (TTC, Sigma) at 37℃ for 30 min to distinguish the infarct area (unstained) from the area at risk (AAR, brick red stained). TTC sections were photographed, digitized, and analyzed off-line. The MI size from TTC staining sections was assessed, as described previously (Ahmet et al., 2005) using NIH Image software. MI size was expresses as a percent of either AAR, or LV.

Statistical evaluation

Date is presented as means ± SEM. Results of experiments were analyzed using One Way Analyses of Variances (ANOVA) with *post hoc* comparison via Bonferroni corrections.

Acknowledgements

This work was fully funded by Intramural Research Program of the National Institute on Aging, National Institutes of Health.

Competing Interests

The authors have no competing interests to declare.

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