

## ORIGINAL RESEARCH

## Activation of the $\beta$ -common receptor by erythropoietin impairs acetylcholine-mediated vasodilation in mouse mesenteric arterioles

Cody R. Kilar<sup>1</sup>, YanPeng Diao<sup>1</sup>, Larysa Sautina<sup>1</sup>, Sivakumar Sekharan<sup>3</sup>, Shahar Keinan<sup>3</sup>, Bianca Carpino<sup>1</sup>, Kirk P. Conrad<sup>2,4</sup>, Rajesh Mohandas<sup>1,5</sup> & Mark S. Segal<sup>1,5</sup>

1 Division of Nephrology, Hypertension, and Transplantation, College of Medicine, University of Florida, Gainesville, Florida

2 Department of Physiology and Functional Genomics, College of Medicine, University of Florida, Gainesville, Florida

3 Cloud Pharmaceuticals, Inc. 6 Davis Dr, Research Triangle Park, North Carolina

4 Department of Obstetrics and Gynecology, College of Medicine, University of Florida, Gainesville, Florida

5 North Florida/South Georgia Veterans Health System, Gainesville, Florida

### Keywords

CD131, erythropoietin, hypertension,  $\beta$ -common receptor.

### Correspondence

Mark S. Segal, Division of Nephrology, Hypertension, and Transplantation, College of Medicine, University of Florida, CG-98, 1600 Archer Road, Gainesville, FL 32610.

Tel: 352 273 8820

Fax: 352 392 5465

E-mail: segalms@medicine.ufl.edu

### Present address

Sivakumar Sekharan, The Cambridge Crystallographic Data Centre, 174 Frelinghuysen Road, Piscataway, New Jersey 08854

Received: 29 January 2018; Revised: 26 May 2018; Accepted: 29 May 2018

doi: 10.14814/phy2.13751

*Physiol Rep* 6 (12), 2018, e13751,  
<https://doi.org/10.14814/phy2.13751>

### Abstract

Clinically, erythropoietin (EPO) is known to increase systemic vascular resistance and arterial blood pressure. However, EPO stimulates the production of the potent vasodilator, nitric oxide (NO), in culture endothelial cells. The mechanism by which EPO causes vasoconstriction despite stimulating NO production may be dependent on its ability to activate two receptor complexes, the homodimeric EPO (EPOR<sub>2</sub>) and the heterodimeric EPOR/ $\beta$ -common receptor ( $\beta$ CR). The purpose of this study was to investigate the contribution of each receptor to the vasoactive properties of EPO. First-order, mesenteric arteries were isolated from 16-week-old male C57BL/6 mice, and arterial function was studied in pressure arteriographs. To determine the contribution of each receptor complex, EPO-stimulating peptide (ESP), which binds and activates the heterodimeric EPOR/ $\beta$ CR complex, and EPO, which activates both receptors, were added to the arteriograph chamber 20 min prior to evaluation of endothelium-dependent (acetylcholine, bradykinin, A23187) and endothelium-independent (sodium nitroprusside) vasodilator responses. Only ACh-induced vasodilation was impaired in arteries pretreated with EPO or ESP. EPO and ESP pretreatment abolished ACh-induced vasodilation by 100% and 60%, respectively. EPO and ESP did not affect endothelium-independent vasodilation by SNP. Additionally, a novel  $\beta$ CR inhibitory peptide ( $\beta$ IP), which was computationally developed, prevented the impairment of acetylcholine-induced vasodilation by EPO and ESP, further implicating the EPOR/ $\beta$ CR complex. Last, pretreatment with either EPO or ESP did not affect vasoconstriction by phenylephrine and KCl. Taken together, these findings suggest that acute activation of the heterodimeric EPOR/ $\beta$ CR in endothelial cells leads to a selective impairment of ACh-mediated vasodilator response in mouse mesenteric resistance arteries.

### Introduction

Since the introduction of recombinant erythropoietin (EPO) in the 1980s for treatment of anemia, it has become one of the most widely used cytokines in clinical practice (Jelkmann 1986; Tögel et al. 2016). A common adverse effect of EPO administration is elevation of

arterial blood pressure and increase in systemic vascular resistance (Drüeke et al. 2006; Singh et al. 2006; Liu et al. 2011). While several factors, particularly an increase in erythrocyte mass, were initially postulated to cause EPO-induced hypertension (Martin and Moncada 1988; Fishbane and Besarab 2007), a series of experiments by Vaziri et al. (1995) demonstrated that the hypertensive effects of

EPO are independent of its erythropoietic action. While the erythropoietic action of EPO is mediated by the homodimeric EPO receptor (EPOR<sub>2</sub>), EPO also activates a heterodimeric EPOR/ $\beta$ -common receptor ( $\beta$ CR), which is believed to be responsible for the tissue protective effects of EPO (Leist et al. 2004; Sautina et al. 2010; Coldewey et al. 2013; Yang et al. 2013). On one hand, EPO stimulates production of the potent vasodilator, nitric oxide (NO), in cultured bone marrow-derived angiogenic cells (BMDACs) (Sautina et al. 2010) and endothelial cells (Su et al. 2011), via activation of the heterodimeric EPOR/ $\beta$ CR (Sautina et al. 2010). On the other hand, EPO also induces the release of the potent vasoconstrictor endothelin-1 (ET-1) in endothelial cells in vitro (Carlini et al. 1993a,b). EPO also directly acts on vascular smooth muscle cells to mobilize calcium and facilitate contraction (Morakkabati et al. 1996). To date, however, it has not been determined if the release of endothelin-1 and effects on smooth muscle cell are mediated by the EPOR<sub>2</sub> or the EPOR/ $\beta$ CR. Interestingly, in healthy human subjects, EPO administered intravenously into the brachial artery over a large concentration range impaired cyclooxygenase-dependent vasodilation by acetylcholine in the forearm resistance vasculature without changing in blood pressure (Wada et al. 1999). To our knowledge, there has not been a study to date which identifies whether activation of the homodimeric EPOR<sub>2</sub>, the heterodimeric EPOR/ $\beta$ CR, or both is responsible for the vasoactive effects observed. The aim of this study was to evaluate whether the acute vasoactive effects of EPO are mediated by the homodimeric EPOR<sub>2</sub>, the heterodimeric EPOR/ $\beta$ CR, or both. To this end, we developed a novel  $\beta$ CR inhibitory peptide ( $\beta$ IP) (Kilar et al. 2018), which was extracted from a portion of helix-A from a mutant EPO (PDB code: 1EER, chain A). We introduced a double mutation at sites 7 and 11 to the 16-amino acid from the extracted portion of EPO and found the amino acid peptide model comprising of VLERYLHEAKHAEKIT decreases bioavailable nitric oxide and angiogenic potential of the  $\beta$ CR (Kilar et al. 2018). The use of this peptide allows us to study, in isolation, the effects of EPO on the heterodimer EPOR/ $\beta$ CR.

## Methods

The utilization of mice in this study was approved by the Institutional Animal Care and Use Committee at University of Florida and was in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (revised 1996). Mice were housed in a 12:12-h light/dark cycle and given access to standard mouse chow and water ad libitum.

## Drugs

Acetylcholine (ACh), bradykinin (BK), A23187, sodium nitroprusside (SNP), Tempol, phenylephrine (PE), and potassium chloride (KCl) were obtained from Sigma-Aldrich (St. Louis, MO). Human recombinant erythropoietin (EPO) was purchased from Zydus (Cadila, India). EPO-stimulating peptide (ESP) derived from a 11-amino acid peptide sequence of the helix B of EPO which interacts with the  $\beta$ CR (Brines et al. 2008) and the  $\beta$ CR inhibitory peptide ( $\beta$ IP) were synthesized by the core facility (Kilar et al. 2018).

## Isobaric arteriography

Sixteen-week-old male C57BL/6 mice were anesthetized with 5% isoflurane/O<sub>2</sub> mix and euthanized by exsanguination. The abdominal cavity was opened to expose the mesenteric cascade, which was then isolated and placed in ice-cold HEPES-buffered physiological saline solution (a modified Krebs buffer composed of sodium chloride 142 mmol/L, potassium chloride 4.7 mmol/L, magnesium sulfate 1.17 mmol/L, calcium chloride 2.5 mmol/L, potassium phosphate 1.18 mmol/L, HEPES 10 mmol/L, and glucose 5.5 mmol/L) and maintained at a pH of 7.4 at 37°C. With the aid of a dissecting microscope, first-order, mesenteric arterioles were isolated and removed from the surrounding fat as previously described (Novak et al. 2002). The mesenteric arterioles (unpressurized inner diameter, 100–200  $\mu$ m) were then transferred to a pressure arteriograph system (Living Systems, Burlington, VT).

## Evaluation of endothelium-dependent and endothelium-independent vasodilation

Mesenteric arteries were pretreated with EPO 50 mIU/mL, ESP 25 ng/mL, or vehicle for 20 min and precontracted with PE to 50% initial diameter before vasodilatory responses were assessed through the cumulative addition of endothelium-dependent vasodilators ACh ( $10^{-9}$ – $10^{-4}$  mol/L), BK ( $10^{-9}$ – $10^{-4}$  mol/L), A23187 ( $10^{-9}$ – $10^{-4}$  mol/L), and endothelium-independent vasodilator SNP ( $10^{-9}$ – $10^{-4}$  mol/L) to the arteriography chamber. Our unpublished data demonstrate that ACh, BK, and A23187 elicit endothelium-dependent vasodilatory effects in first-order mouse mesenteric vessels through an eNOS-dependent pathway. Vasodilation was calculated according to the following formula:

$$\text{Relaxation}(\%) = (D_s - D_b)/(D_m - D_b) \times 100$$

where  $D_m$  is the maximal inner diameter recorded at 60 mmHg in calcium-free HEPES-buffered PSS

containing EGTA and papaverine to inhibit vascular smooth muscle function,  $D_s$  is the steady-state inner diameter recorded after each addition of the drug, and  $D_b$  is the initial baseline inner diameter recorded immediately before the first addition of the vasodilatory agent.

### Evaluation of vasoconstrictor responses

To determine whether acute pretreatment with EPO, ESP, or vehicle altered vasoconstrictor responsiveness in mesenteric arterioles, concentration–response curves to PE ( $10^{-9}$ – $10^{-4}$  mol/L) and depolarizing concentration of  $K^+$  (0 mmol/L–100 mmol/L) were evaluated as previously described (Muller-Delp et al. 2002). Briefly, intraluminal diameters were measured in micrometers at 60 mmHg and expressed as a percentage of vasoconstriction as follows:

$$\text{Vasoconstriction}(\%) = (D_b - D_s) / D_b \times 100$$

where  $D_b$  was the initial baseline intraluminal diameter measured before experimental intervention and  $D_s$  was the steady-state intraluminal diameter measured after agonist addition.

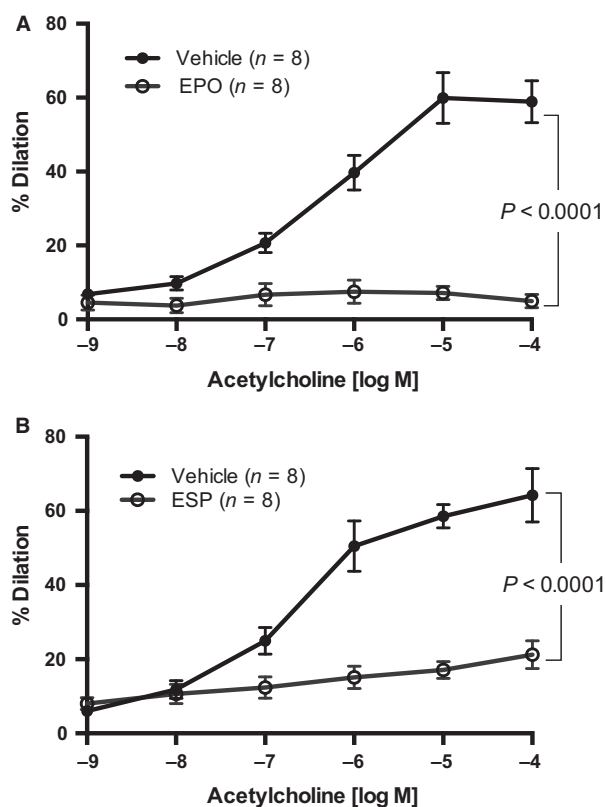
### Statistical and data analysis

Two-way repeated-measures ANOVA was used to detect differences between (vehicle vs. treatment) and within (drug concentrations) factors for the mesenteric resistance artery studies (GraphPad Software, La Jolla, CA). All data are presented as means  $\pm$  SE. In all statistical analyses,  $n$  indicates the number of animals in each group. Student's  $t$ -tests were performed to identify differences between the maximal responses ( $E_{\max}$ ) to ACh, SNP, BK, A23187, KCl, and PE to identify differences in the amount of dilation with respect to intervention, and to determine differences in sensitivity ( $EC_{50}$ ) to ACh, SNP, BK, A23187, KCl, and PE. Significance was defined as  $P \leq 0.05$ .

## Results

### EPO and ESP impair ACh-mediated endothelium-dependent vasodilation but not endothelium-independent vasodilation by SNP

ACh elicited a maximum vasodilation of ~60% in first-order mesenteric arterioles (Fig. 1). However, in vessels pretreated with 50 mIU/mL EPO, the ACh-induced vasodilation was virtually abolished (Fig. 1A). Pretreatment with 25 ng/mL ESP also impaired ACh-induced vasodilation (Fig. 1B), but to a lesser extent than EPO (by ~60% at  $10^{-4}$  mol/L, respectively). Importantly, acute pretreatment with EPO or ESP did not alter endothelial-independent vasodilation, maximal

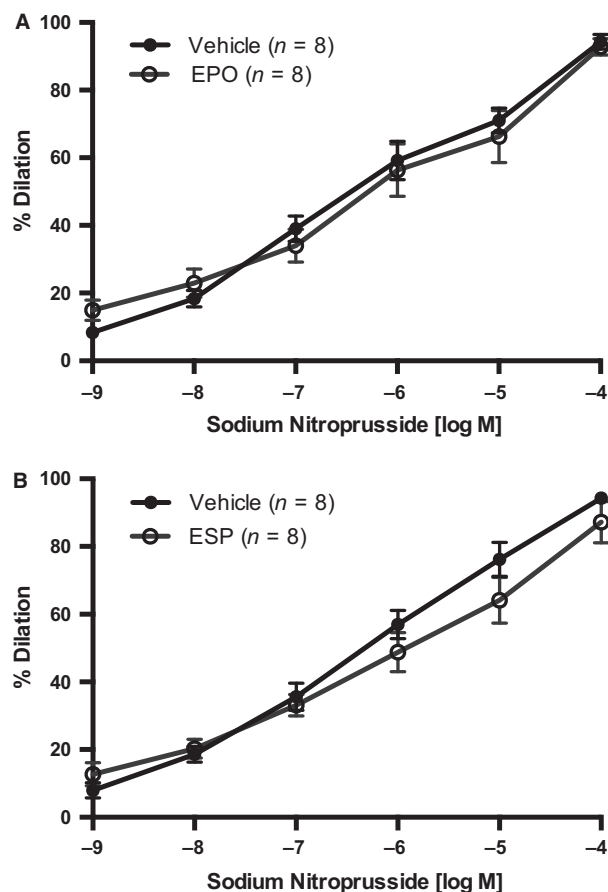


**Figure 1.** Effect of EPO or the ESP on ACh-induced vasodilation of first-order mesenteric arterioles. First-order mesenteric arteries were acutely pretreated with (A) EPO (50 mIU/mL) or (B) ESP (25 ng/mL) for 20 min before assessment of ACh-induced endothelium-dependent vasodilation. All values are means  $\pm$  SE.  $n$  equals the number of animals studied.

response ( $E_{\max}$ ) or sensitivity ( $EC_{50}$ ) to SNP (Fig. 2), indicating that the two peptides did not interfere with the intrinsic ability of the vascular smooth muscle to vasodilate in response to NO. Whether the vessels were obtained from male or female mice, the response to ESP treatment was similar (results not shown).

### EPO or ESP-induced impairment of mesenteric resistance artery endothelium-dependent relaxation to ACh is not caused by an increase in oxidative stress

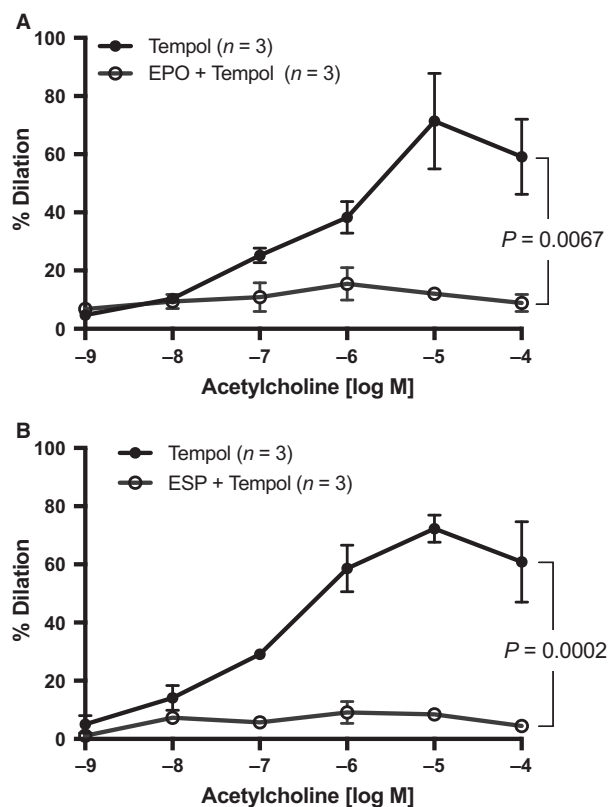
To test whether the compromise of ACh-induced endothelium-dependent vasodilation by EPO and ESP was due to increased reactive oxygen species as previously reported (Chen et al. 1997), we employed the  $O_2^-$  scavenger Tempol (5  $\mu$ mol/L). Pretreatment with Tempol for 20 min prior to EPO or ESP administration did not affect the inhibition of ACh-induced vasodilation by EPO (Fig. 3A) or ESP (Fig. 3B).



**Figure 2.** Effect of EPO and the ESP sodium nitroprusside (SNP)-induced vasodilation. First-order mesenteric arteries were acutely pretreated with (A) EPO (50 mIU/mL) or (B) ESP (25 ng/mL) for 20 min before assessment of SNP-induced endothelium-independent vasodilation. All values are means  $\pm$  SE. *n* equals the number of animals studied. Responses were not different between groups.

### A23187 and bradykinin-induced endothelium-dependent vasodilation is unaffected by acute pretreatment with EPO or ESP

To determine whether the effect of EPO and ESP was unique to ACh, we investigated bradykinin and A23187 – receptor-mediated and nonreceptor-mediated endothelium-dependent vasodilators, respectively. On one hand, ACh and bradykinin receptors both utilize G-protein coupled inositol-1,4,5-trisphosphate ( $IP_3$ ) signaling cascade in endothelial cells to release calcium from endoplasmic reticulum that, in turn, activates  $K^+$  channels leading to hyperpolarization and calcium influx (Brenner et al. 1989). On the other hand, A23187 directly increases cell permeability to calcium, thereby stimulating eNOS. Pretreatment of first-order mesenteric arterioles with EPO or



**Figure 3.** Tempol did not restore ablated ACh-induced endothelium-dependent vasodilation. First-order mesenteric arteries were acutely pretreated with (A) EPO (50 mIU/mL) or (B) ESP (25 ng/mL) for 20 min in addition to Tempol (5  $\mu$ mol/L) before assessment of ACh-induced endothelium-dependent vasodilation. All values are means  $\pm$  SE. *n* equals the number of animals studied.

an ESP had no effect on A23187- or bradykinin-induced endothelium-dependent vasodilation suggesting that EPO inhibits ACh-induced vasodilation at the level of muscarinic receptor or muscarinic receptor-G protein interaction (Fig. 4A and B). Additionally, ( $EC_{50}$ ) and maximal response ( $E_{max}$ ) were unaffected in vessels pretreated with EPO or ESP when compared with controls in both A23187- or BK-induced vasodilation.

### $\beta$ IP prevents inhibition of ACh-induced endothelium-dependent vasodilation by EPO or ESP

Pretreatment of mesenteric arteries with  $\beta$ IP blocked the inhibition of ACh-induced vasodilation in vessels by EPO (Fig. 5A) or ESP (Fig. 5B). Additionally, there was also no significant differences between the sensitivity of EPO ( $EC_{50} = 1.758 \times 10^{-6}$ ) or ESP ( $EC_{50} = 2.017 \times 10^{-6}$ ) to ACh-induced vasodilation. Moreover, the maximal responses ( $E_{max}$ ) were also not significantly different.

## EPO or ESP does not alter receptor-dependent or receptor-independent vasoconstriction

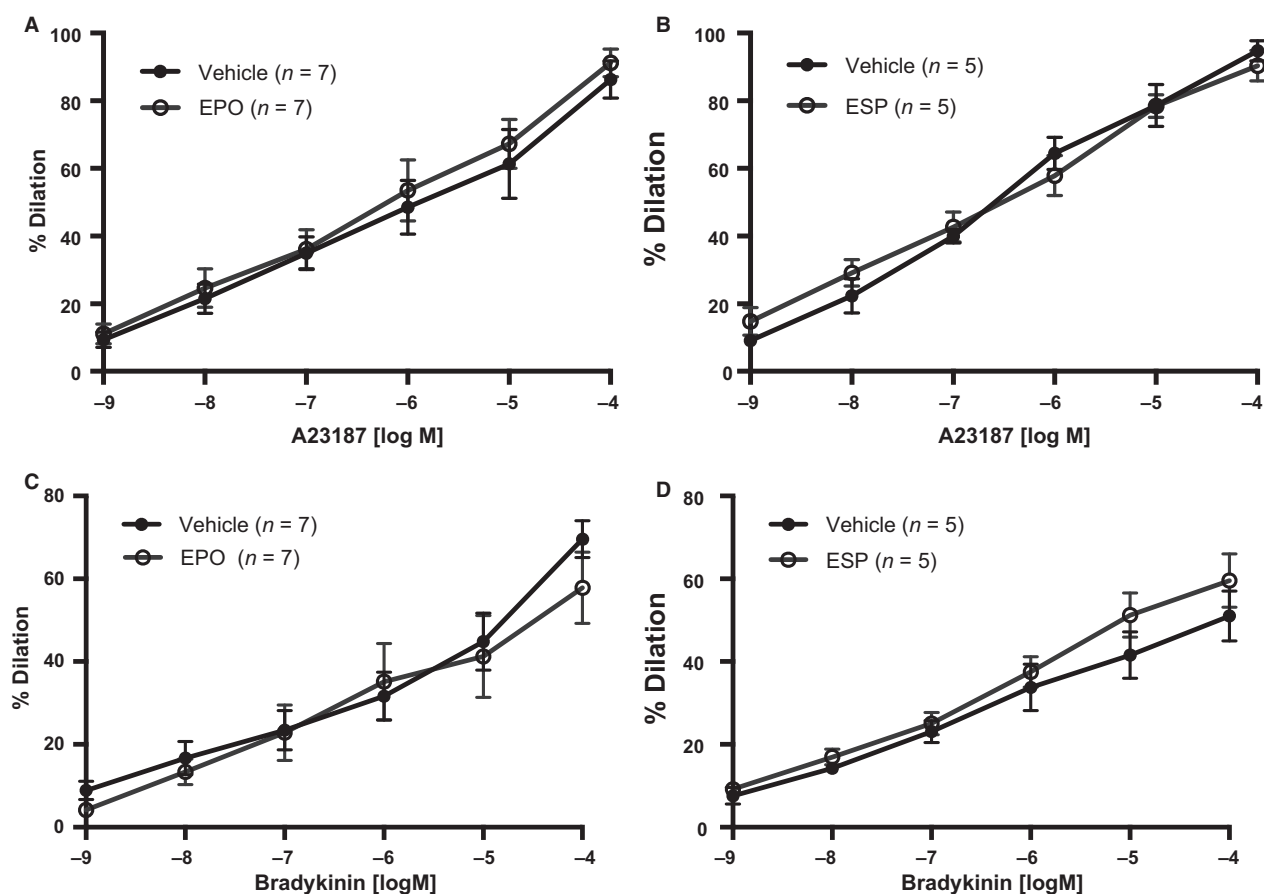
Because it has been reported that EPO increases production of potent vasoconstrictors, such as endothelin-1 (Vogel et al. 1997; Barhoumi et al. 2014), we explored whether acute pretreatment with EPO or ESP would potentiate vasoconstriction in first-order mesenteric arteries. Neither EPO nor ESP, however, affected vasoconstriction by PE (Fig. 6A and C) or KCl (Fig. 6B and D). Additionally, EPO or ESP did not alter the sensitivity ( $EC_{50}$ ) or maximal response ( $E_{max}$ ) of the vasoconstrictors PE or KCl.

## Discussion

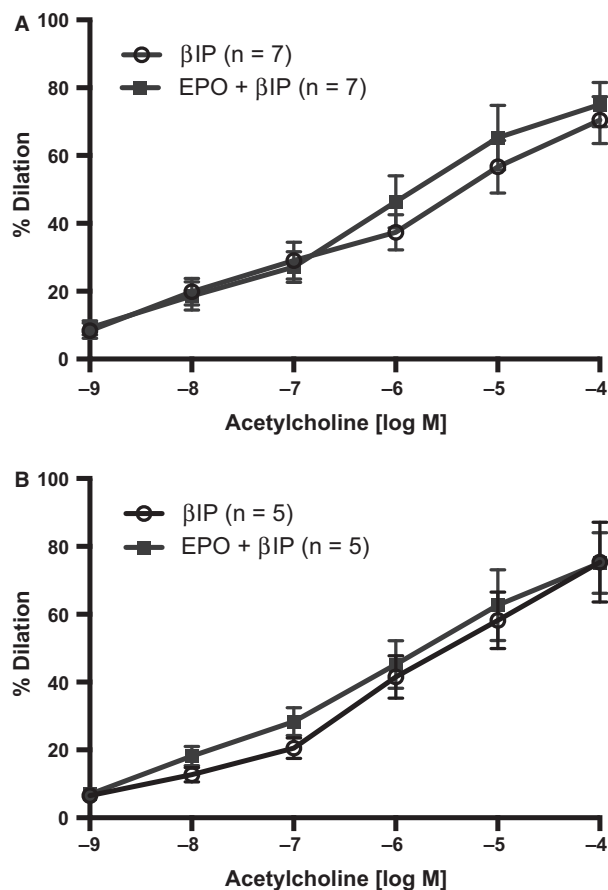
There are several new findings that emerged from this research: (1) acute activation of the  $\beta$ CR with EPO or

ESP in mouse mesenteric arteries inhibited ACh, but not BK or A23187 endothelium-dependent vasodilation, and this inhibition of ACh was blocked by a novel  $\beta$ CR inhibitor,  $\beta$ IP; (2) impaired ACh-induced vasodilation by EPO and ESP did not appear to be due to generation of reactive oxygen species; and (3) activation of the  $\beta$ CR by either EPO or ESP did not affect mesenteric vessel vasoconstriction to PE and KCl. Our results suggest that activation of the  $\beta$ CR on the vascular endothelium with EPO or ESP specifically impairs endothelial vasodilatory responses to ACh, which may contribute to the pathogenesis of EPO-induced hypertension.

Animal models have been utilized to try to investigate the clinical observation of EPO-induced hypertension (Vaziri et al. 1995, 1996; Schiffel and Lang 1997; Vaziri 1999). Animals given high doses of EPO exhibit a marked increase in hemoglobin concentration (Vaziri 1999; Singh et al. 2006; Fishbane and Besarab 2007), as well as increased systemic vascular resistance (Becker et al. 2016)



**Figure 4.** EPO or ESP had no effect on endothelium-dependent relaxation to A23187 or BK. First-order mesenteric arteries subjected to acute pretreatment of EPO (50 mIU/mL) (A, C) or an ESP (25 ng/mL) (B, D) before endothelium-dependent vasodilators A23187 and BK were assessed. All values are means  $\pm$  SE. *n* equals the number of animals studied.



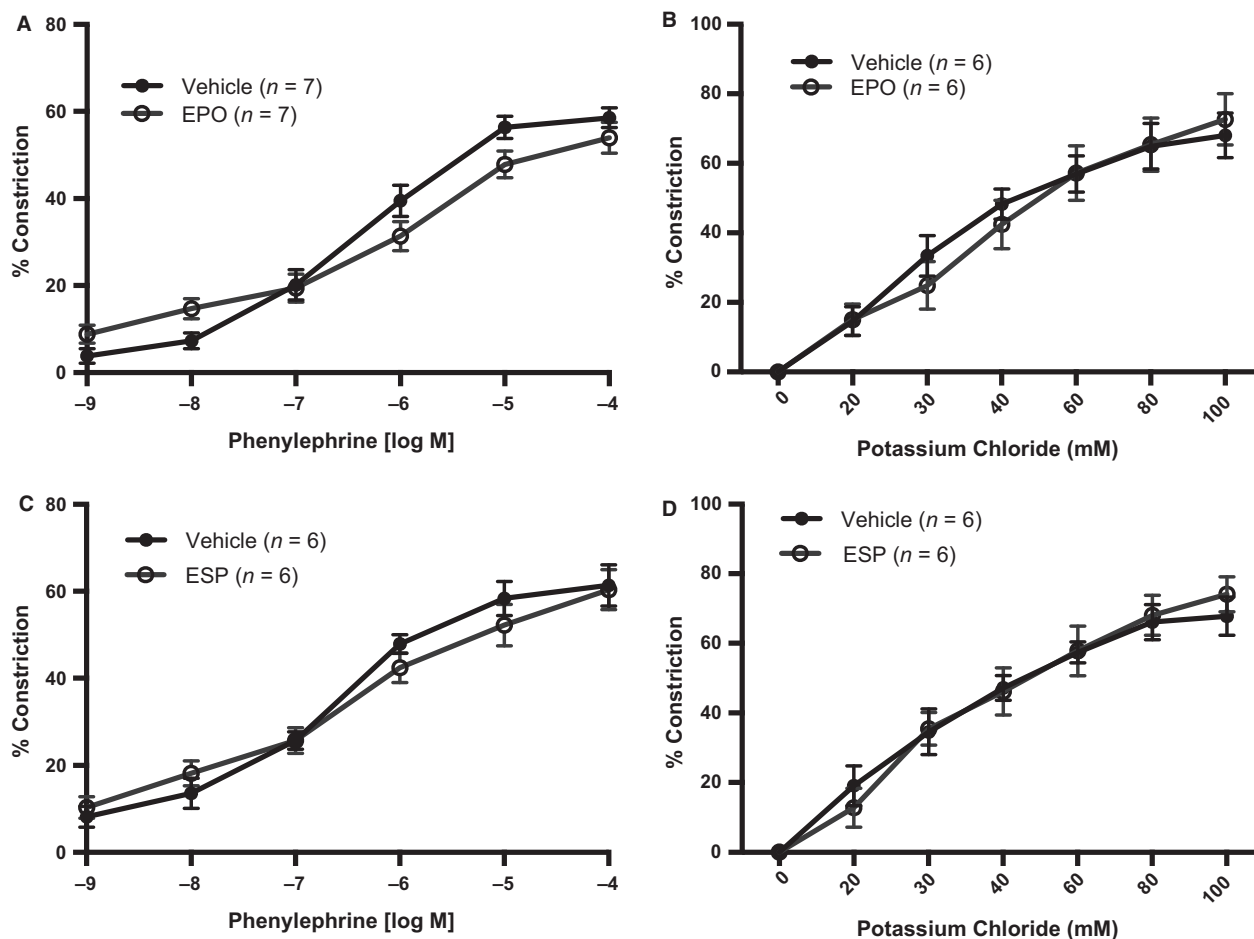
**Figure 5.** Novel  $\beta$ IP restores ablated ACh-induced endothelium-dependent vasodilation. First-order mesenteric arteries were treated with the  $\beta$ IP (10  $\mu$ M) for 30 min prior to being acutely pretreated with (A) EPO (50 mIU/mL) or (B) ESP (25 ng/mL) for 20 min. ACh-induced endothelium-dependent vasodilation was then assessed. Sensitivity of EPO ( $EC_{50} = 1.758 \times 10^{-6}$ ) or ESP ( $EC_{50} = 2.017 \times 10^{-6}$ ) was not reduced when compared with vehicle or when compared with one another. All values are means  $\pm$  SE.  $n$  equals the number of animals studied. Responses were not different between groups.

and blood pressure (Buemi et al. 1995; Kanbay et al. 2007; Becker et al. 2016) similar to patients. In addition to increased systemic vascular resistance and blood pressure, small resistance arteries demonstrate diminished endothelium-dependent vasodilation to ACh (Buemi et al. 1995; Noguchi et al. 2001; Annuk et al. 2006; Briet et al. 2013). However, there are no reports on whether EPO treatment differentially affects receptor- and nonreceptor-mediated endothelium-dependent vasodilation, and if so, which of the EPO receptor dimeric complexes might be involved. Our data demonstrate that after short-term incubation with EPO or an ESP, ACh-induced vasodilation is selectively impaired via the EPOR/ $\beta$ CR heterodimeric receptor (Fig. 1).

Briet et al. (2013) demonstrated that oxidative stress plays a role in EPO-induced hypertension in humans, and in gluteal subcutaneous resistance arteries isolated from these patients, Tempol restored endothelial function. However, in our model, Tempol did not ameliorate the inhibition of ACh-mediated vasodilation by EPO or ESP in isolated mouse mesenteric arteries (Fig. 3), suggesting that the impairment was not due to increased reactive oxygen species. Although there are many potential explanations for these discrepant results, one possibility is the marked difference in the duration of arterial exposure to EPO (months in vivo vs. minutes in vitro).

We further demonstrated an essential role for the  $\beta$ CR in the impairment of ACh-mediated vasodilation by EPO, insofar as ESP, which specifically agonizes the  $\beta$ CR/EPOR<sub>2</sub> heterodimer and does not stimulate erythropoiesis, duplicated the inhibitory action of EPO, albeit perhaps to a lesser degree. To our knowledge, there are limited reports of the involvement of this receptor in endothelial function (Su et al. 2011). We confirmed the critical role of  $\beta$ CR by using a novel inhibitor of  $\beta$ CR ( $\beta$ IP), which acts as a competitive inhibitor of ligand binding to  $\beta$ CR.  $\beta$ IP abrogated the ability of EPO and ESP to inhibit the vasodilatory effect of ACh. Interestingly, EPO and ESP had similar sensitivities ( $EC_{50}$ ) to ACh after treatment with  $\beta$ IP, suggesting that activation of the  $\beta$ CR is responsible for decreasing the vasoactive response to ACh.

The mechanisms by which EPO compromises ACh-mediated endothelium-dependent vasodilation is unknown. Using immunoprecipitation and immunofluorescence, our group reported co-localization of  $\beta$ CR and vascular endothelial growth factor receptor 2 (VEGFR-2) in human bone marrow-derived angiogenic cells (BMDACs) (Sautina et al. 2010), and other investigators also reported that muscarinic acetylcholine receptor (mAChR) has a physical interaction with VEGFR-2 in human neuroblastoma cells (Edelstein et al. 2011). Furthermore, both VEGFR-2 and mAChR have been shown to localize in caveolae (Rybin et al. 2000; Cho et al. 2004; Sonveaux et al. 2004; Lampugnani et al. 2006). Taken together, these findings raise the possibility that  $\beta$ CR and the muscarinic acetylcholine receptor 2 (mAChR2) are co-localized in the endothelial caveolae. It has been suggested that once activated, the  $\beta$ CR is internalized to elicit its downstream responses (Debeljak et al. 2014). However, there is no experimental evidence to support this mechanism. Further studies are needed to determine if  $\beta$ CR is associated with the caveolae in endothelial cells, and if so, whether activation leads to internalization. How this might lead to impairment of ACh-mediated endothelium-dependent vasodilation also requires further investigation.



**Figure 6.** Comparison of PE (A, C) and KCl (B, D) constrictor responses with acute (20-min) pretreatment of EPO (50 mIU/mL) (A, B) and the ESP (25 ng/mL) (C, D). All values are means  $\pm$  SE. *n* equals the number of animals studied. Responses were not different between groups.

Carlini et al. (1993b) have shown that animals treated with EPO have increased expression of ET-1 (Su et al. 2011). Also, EPO administration to patients with anemia lead to increased production of cyclooxygenase-dependent vasoconstrictors in forearm-resistance arteries (Carlini et al. 1993a). Bode-Böger et al. (1996) described that acute EPO treatment significantly enhanced norepinephrine-induced vasoconstriction in rabbit aorta and carotid arteries. However, our data suggest that after acute treatment in vitro, EPO does not potentiate vasoconstrictor responses in mouse mesenteric arteries or reduce baseline internal diameter, thus mitigating against a role for cyclooxygenase-generated vasoconstrictors. One major difference between the studies described above and this current study is the concentration of EPO used for experimentation, which was 100–1000 times the concentration that was used in this study (Bode-Böger et al. 1996).

In summary, we demonstrated that activation of the  $\beta$ CR leads to impairment of ACh-induced vasodilation,

which is prevented by a novel  $\beta$ IP. To our knowledge, this is the first study showing that activation of the  $\beta$ CR affects vasoreactivity. In the current literature, little evidence exists for the role of the  $\beta$ CR on the vasculature (Bohr et al. 2013).  $\beta$ CR knockout mice appears to have normal development and, as would be expected, normal erythropoiesis (Sautina et al. 2010; Hand and Brines 2011; Cerami 2012; Debeljak et al. 2014; Collino et al. 2015). However, investigation of blood pressure and arterial function has not been reported. It would also be interesting to test whether  $\beta$ IP attenuates EPO-induced hypertension in experimental animal models.

## Acknowledgments

This work was supported by NIH/NIGMS P50 GM111152 (MSS), NIH K08 HL130945, and by the Gatorade Trust through funds distributed by the University of Florida, Department of Medicine and Division of Nephrology, Hypertension, & Transplantation.

## Conflict of Interest

None declared.

## References

- Annuk, M., T. Linde, L. Lind, and B. Fellström. 2006. Erythropoietin impairs endothelial vasodilatory function in patients with renal anemia and in healthy subjects. *Nephron Clin. Pract.* 102:c30–34.
- Barhoumi, T., M. Briet, D. A. Kasal, J. C. Fraulob-Aquino, N. Idris-Khodja, P. Laurant, et al. 2014. Erythropoietin-induced hypertension and vascular injury in mice overexpressing human endothelin-1: exercise attenuated hypertension, oxidative stress, inflammation and immune response. *J. Hypertens.* 32:784–794.
- Becker, J., E. Peter-Thiebaut, L. El Fertak, M. Denu, H. Jacobs, P. Reilly, et al. 2016. Erythropoietin recapitulates hemodynamic features of hypoxia-induced pulmonary hypertension in mice. *Eur. Respiratory Soc.* 48:PA5097.
- Bode-Böger, S. M., R. H. Böger, M. Kuhn, J. Radermacher, and J. C. Frölich. 1996. Recombinant human erythropoietin enhances vasoconstrictor tone via endothelin-1 and constrictor prostanoids. *Kidney Int.* 50:1255–1261.
- Bohr, S., S. J. Patel, K. Shen, A. G. Vitalo, M. Brines, A. Cerami, et al. 2013. Alternative erythropoietin-mediated signaling prevents secondary microvascular thrombosis and inflammation within cutaneous burns. *Proc. Natl Acad. Sci. USA* 110:3513–3518.
- Brenner, B. M., J. L. Troy, and B. J. Ballermann. 1989. Endothelium-dependent vascular responses. *Mediators and mechanisms.* *J. Clin. Invest.* 84:1373–1378.
- Briet, M., T. Barhoumi, M. O. Mian, C. Sierra, P. Boutouyrie, M. Davidman, et al. 2013. Effects of recombinant human erythropoietin on resistance artery endothelial function in stage 4 chronic kidney disease. *J. Am. Heart Assoc.* 2:e000128.
- Brines, M., N. S. Patel, P. Villa, C. Brines, T. Mennini, M. De Paola, et al. 2008. Nonerythropoietic, tissue-protective peptides derived from the tertiary structure of erythropoietin. *Proc Natl Acad Sci U S A* 105:10925–10930.
- Buemi, M., G. Denuzzo, A. Allegra, C. Aloisi, F. Squadrito, G. Squadrito, et al. 1995. Recombinant human erythropoietin inhibits the cutaneous vasodilatation induced by acetylcholine. *Int. J. Microcirc. Clin. Exp.* 15:283–286.
- Carlini, R., C. I. Obialo, and M. Rothstein. 1993a. Intravenous erythropoietin (rHuEPO) administration increases plasma endothelin and blood pressure in hemodialysis patients. *Am. J. Hypertens.* 6:103–107.
- Carlini, R. G., A. S. Dusso, C. I. Obialo, U. M. Alvarez, and M. Rothstein. 1993b. Recombinant human erythropoietin (rHuEPO) increases endothelin-1 release by endothelial cells. *Kidney Int.* 43:1010–1014.
- Cerami, A. 2012. TNF and EPO: major players in the innate immune response: their discovery. *Ann. Rheum. Dis.* 71 (Suppl 2):i55–59.
- Chen, H. C., J. C. Tsai, J. H. Tsai, and Y. H. Lai. 1997. Recombinant human erythropoietin enhances superoxide production by FMLP-stimulated polymorphonuclear leukocytes in hemodialysis patients. *Kidney Int.* 52:1390–1394.
- Cho, C. H., C. S. Lee, M. Chang, I. H. Jang, S. J. Kim, I. Hwang, et al. 2004. Localization of VEGFR-2 and PLD2 in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK. *Am. J. Physiol. Heart Circ. Physiol.* 286:H1881–1888.
- Coldewey, S. M., A. I. Khan, A. Kapoor, M. Collino, M. Rogazzo, M. Brines, et al. 2013. Erythropoietin attenuates acute kidney dysfunction in murine experimental sepsis by activation of the  $\beta$ -common receptor. *Kidney Int.* 84:482–490.
- Collino, M., C. Thiemermann, A. Cerami, and M. Brines. 2015. Flipping the molecular switch for innate protection and repair of tissues: Long-lasting effects of a non-erythropoietic small peptide engineered from erythropoietin. *Pharmacol. Ther.* 151:32–40.
- Debeljak, N., P. Solár, and A. J. Sytkowski. 2014. Erythropoietin and cancer: the unintended consequences of anemia correction. *Front Immunol.* 5:563.
- Drüeke, T. B., F. Locatelli, N. Clyne, K. U. Eckardt, I. C. Macdougall, D. Tsakiris, et al. 2006. Normalization of hemoglobin level in patients with chronic kidney disease and anemia. *N. Engl. J. Med.* 355:2071–2084.
- Edelstein, J., T. Hao, Q. Cao, L. Morales, and P. Rockwell. 2011. Crosstalk between VEGFR2 and muscarinic receptors regulates the mTOR pathway in serum starved SK-N-SH human neuroblastoma cells. *Cell. Signal.* 23:239–248.
- Fishbane, S., and A. Besarab. 2007. Mechanism of increased mortality risk with erythropoietin treatment to higher hemoglobin targets. *Clin. J. Am. Soc. Nephrol.* 2:1274–1282.
- Hand, C. C., and M. Brines. 2011. Promises and pitfalls in erythropoietin-mediated tissue protection: are nonerythropoietic derivatives a way forward? *J. Investig. Med.* 59:1073–1082.
- Jelkmann, W. 1986. Erythropoietin research, 80 years after the initial studies by Carnot and Deflandre. *Respir. Physiol.* 63:257–266.
- Kanbay, M., A. Akcay, T. Delibasi, B. Uz, A. Kaya, C. Koca, et al. 2007. Comparison of effects of darbepoetin alfa and epoetin alfa on serum endothelin level and blood pressure. *Adv. Ther.* 24:346–352.
- Kilar, C. R., S. Sekharan, L. Sautina, Y. Diao, S. Keinan, Y. Shen, et al. 2018. Computational design and experimental characterization of a novel  $\beta$ -common receptor inhibitory peptide. *Peptides* 104:1–6.



- Lampugnani, M. G., F. Orsenigo, M. C. Gagliani, C. Tacchetti, and E. Dejana. 2006. Vascular endothelial cadherin controls VEGFR-2 internalization and signaling from intracellular compartments. *J. Cell Biol.* 174:593–604.
- Leist, M., P. Ghezzi, G. Grasso, R. Bianchi, P. Villa, M. Fratelli, et al. 2004. Derivatives of erythropoietin that are tissue protective but not erythropoietic. *Science* 305:239–242.
- Liu, Y., Y. Xu, F. Thilo, U. G. Friis, B. L. Jensen, A. Scholze, et al. 2011. Erythropoietin increases expression and function of transient receptor potential canonical 5 channels. *Hypertension* 58:317–324.
- Martin, J., and S. Moncada. 1988. Blood pressure, erythropoietin, and nitric oxide. *Lancet* 1:644.
- Morakkabati, N., F. Gollnick, R. Meyer, J. Fandrey, and W. Jelkmann. 1996. Erythropoietin induces  $Ca^{2+}$  mobilization and contraction in rat mesangial and aortic smooth muscle cultures. *Exp. Hematol.* 24:392–397.
- Muller-Delp, J., S. A. Spier, M. W. Ramsey, L. A. Lesniewski, A. Papadopoulos, J. D. Humphrey, et al. 2002. Effects of aging on vasoconstrictor and mechanical properties of rat skeletal muscle arterioles. *Am. J. Physiol. Heart Circ. Physiol.* 282:H1843–1854.
- Noguchi, K., S. Yamashiro, T. Matsuzaki, M. Sakanashi, J. Nakasone, K. Miyagi, et al. 2001. Effect of 1-week treatment with erythropoietin on the vascular endothelial function in anaesthetized rabbits. *Br. J. Pharmacol.* 133:395–405.
- Novak, J., R. J. Ramirez, R. E. Gandley, O. D. Sherwood, and K. P. Conrad. 2002. Myogenic reactivity is reduced in small renal arteries isolated from relaxin-treated rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283:R349–355.
- Rybin, V. O., X. Xu, M. P. Lisanti, and S. F. Steinberg. 2000. Differential targeting of beta -adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J. Biol. Chem.* 275:41447–41457.
- Sautina, L., Y. Sautin, E. Beem, Z. Zhou, A. Schuler, J. Brennan, et al. 2010. Induction of nitric oxide by erythropoietin is mediated by the beta common receptor and requires interaction with VEGF receptor 2. *Blood* 115:896–905.
- Schiffli, H., and S. M. Lang. 1997. Hypertension induced by recombinant human erythropoietin (rHU-EPO) can be prevented by indomethacin. Pathogenetic role of cytosolic calcium. *Eur. J. Med. Res.* 2:97–100.
- Singh, A. K., L. Szczech, K. L. Tang, H. Barnhart, S. Sapp, M. Wolfson, et al. 2006. Correction of anemia with epoetin alfa in chronic kidney disease. *N. Engl. J. Med.* 355:2085–2098.
- Sonveaux, P., P. Martinive, J. DeWever, Z. Batova, G. Daneau, M. Pelat, et al. 2004. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. *Circ. Res.* 95:154–161.
- Su, K. H., S. K. Shyue, Y. R. Kou, L. C. Ching, A. N. Chiang, Y. B. Yu, et al. 2011.  $\beta$  Common receptor integrates the erythropoietin signaling in activation of endothelial nitric oxide synthase. *J. Cell. Physiol.* 226:3330–3339.
- Tögel, F. E., J. D. Ahlstrom, Y. Yang, Z. Hu, P. Zhang, and C. Westenfelder. 2016. Carbamylated erythropoietin outperforms erythropoietin in the treatment of AKI-on-CKD and Other AKI Models. *J. Am. Soc. Nephrol.* 27:3394–3404.
- Vaziri, N. D. 1999. Mechanism of erythropoietin-induced hypertension. *Am. J. Kidney Dis.* 33:821–828.
- Vaziri, N. D., X. J. Zhou, J. Smith, F. Oveisi, K. Baldwin, and R. E. Purdy. 1995. In vivo and in vitro pressor effects of erythropoietin in rats. *Am. J. Physiol.* 269:F838–845.
- Vaziri, N. D., X. J. Zhou, F. Naqvi, J. Smith, F. Oveisi, Z. Q. Wang, et al. 1996. Role of nitric oxide resistance in erythropoietin-induced hypertension in rats with chronic renal failure. *Am. J. Physiol.* 271:E113–122.
- Vogel, V., H. J. Kramer, A. Bäcker, H. Meyer-Lehnert, W. Jelkmann, and J. Fandrey. 1997. Effects of erythropoietin on endothelin-1 synthesis and the cellular calcium messenger system in vascular endothelial cells. *Am. J. Hypertens.* 10:289–296.
- Wada, Y., H. Matsuoka, O. Tamai, K. Kohno, S. Okuda, and T. Imaizumi. 1999. Erythropoietin impairs endothelium-dependent vasorelaxation through cyclooxygenase-dependent mechanisms in humans. *Am. J. Hypertens.* 12:980–987.
- Yang, C., T. Zhao, M. Lin, Z. Zhao, L. Hu, Y. Jia, et al. 2013. Helix B surface peptide administered after insult of ischemia reperfusion improved renal function, structure and apoptosis through beta common receptor/erythropoietin receptor and PI3K/Akt pathway in a murine model. *Exp. Biol. Med.* (Maywood) 238:111–119.