Pharmacological Reports 69 (2017) 658–665

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Short-term exposure of erythropoietin impairs endothelial function through inhibition of nitric oxide production and eNOS mRNA expression in the rat pulmonary artery

Faheem Sultan, Thakur Uttam Singh*, Tarun Kumar, Soya Rungsung, Dipankar Jyoti Rabha, Anamika Vishwakarma, Susanth V. Sukumaran, Arunvikram Kandasamy, Subhashree Parida

Division of Pharmacology and Toxicology, ICAR-Indian Veterinary Research Institute, Izatnagar, 243122 Bareilly, Uttar Pradesh, India

ARTICLE INFO

Article history:
Received 21 October 2016
Received in revised form 30 January 2017
Accepted 3 February 2017
Available online 4 February 2017

Keywords:
Erythropoietin
Pulmonary artery
Endothelial
eNOS
Nitric oxide

ABSTRACT

Background: Administration of recombinant erythropoietin (rEPO) is often associated with systemic and pulmonary arterial hypertension in animals and human. The present study was conducted to determine whether one-week rEPO-treatment can produce any effect on pulmonary vasomotor function.
Methods: Male Wistar rats were injected with rEPO (400 IU/kg *sc*) or saline every other day for one week. Tension, biochemical and Real-Time PCR experiments were conducted on left and right branches of pulmonary artery and main pulmonary artery isolated from the rats.
Results: ACh-induced relaxation was significantly ($p < 0.05$) reduced in rEPO-treated rats in comparison to control animals. Relaxation to the NO donor SNP was not different between the groups. EDHF-induced relaxation was remarkably higher in rEPO-treated group in comparison to control. Phenylephrine-induced contraction was significantly ($p < 0.05$) reduced in rings from rEPO-treated rats at the second and third lowest concentrations of phenylephrine and its potency was not significantly reduced. No significant difference was observed in $CaCl_2$-induced contraction between the groups. Nitric oxide production was significantly reduced in rEPO-treated rats in comparison to control animals. Real-time PCR studies demonstrated a significant decrease ($p < 0.05$) of eNOS transcript. However, peNOS activity was not altered with rEPO treatment.
Conclusion: The present study suggests that EPO-treatment for one week attenuates ACh-stimulated NO production. It does not affect the vasodilatory action of SNP. It showed up-regulation of EDHF and decreased potency of phenylephrine. Thus elevated EPO may diversely affect the vasomotor function of pulmonary artery. Clinically, it is important to observe the use of EPO in hypertensive condition.

Introduction

Erythropoietin is a glycosylated protein secreted from peritubular interstitial cells of the renal cortex [1–3]. It acts on erythroid progenitor cells in the bone marrow and promotes their proliferation and differentiation [4]. Clinically erythropoietin (EPO) is used in severely anemic hemodialysis patients, predialysis patients and patients with tumor chemotherapy-induced anemia. In veterinary medicine, recombinant human erythropoietin (rEPO) is used in canine and feline patients with non-regenerative anemia secondary to chronic renal failure [5–7]. In addition, EPO has been used in blood doping as an antianemic and performance-boosting drug [4,8].

Development of high blood pressure or its aggravation is the most critical adverse effect of rEPO therapy [9] and almost 40% of such patients suffering from end-stage renal disease have pulmonary hypertension [10,11]. Previously it has been reported that upregulation of plasma EPO and its expression in pulmonary vasculature suggests its role in the development of pulmonary arterial hypertension (PAH) [12]. Elevated EPO and polycythemia was shown to be associated with early onset of severe pulmonary arterial hypertension [13]. Hypertension usually occurs within several weeks to months after the onset of EPO treatment [14] and in normotensive subjects EPO was reported to increase mean arterial pressure by 6 mmHg when measured by catheter [15].

* Corresponding author.
E-mail address: tusingh80@gmail.com (T.U. Singh).

http://dx.doi.org/10.1016/j.pharep.2017.02.003

The erythropoietin receptor protein and messenger RNA, classically found in erythroid precursor cells [16], have been described in other cell types, including endothelial cells [17–19]. Under normal physiological conditions vascular tone is maintained by different vasodilators like nitric oxide (NO), prostacyclin (PGI_2), endothelium derived hyperpolarizing factors (EDHFs) and vasoconstrictors like endothelin (ET). Alterations in their endogenous production have been linked to the progression of vascular disorders. Endothelial impairment has been suggested to have a causative role in hypertension development in rats [20] and also in normotensive subjects [21]. Endothelium is affected directly and indirectly by erythropoietin [22].

Using rat as an experimental model, the present study was conducted to determine whether one-week rEPO-treatment can cause any effect on pulmonary vasomotor function. The vascular function was assessed by employing various pharmacological tools like endothelium-dependent vasodilator acetylcholine, endothelium-independent NO donor sodium nitroprusside, NO synthase (NOS) inhibitor L-NAME, cyclooxygenase (COX) inhibitor indomethacin, α_1-adrenergic receptor agonist phenylephrine and $CaCl_2$. We have also used a highly selective inhibitor for inducible NOS (iNOS) *N*-(3-(aminomethyl) benzyl) acetamidine (1400 W) to quantify nitrite formation by estimating nitrite accumulation in the medium after acetylcholine stimulation. Further, we determined the eNOS mRNA expression in pulmonary arterial tissue from control (saline-treated) and rEPO-treated rats.

Materials and methods

Animals

Adult male Wistar rats weighing 150–250 g were used. Animals were kept for an acclimatization period of seven days before the conduction of experiments and were allowed free access to feed and water. All protocols and surgical procedures employed were in accordance with the Institutional Animal Ethics Committee (Approval No. F.1-53/2012-13/JD(R); Dated: 05/03/2015).

Dose and treatment schedule

Animals were divided into two groups i.e. control group and erythropoietin-treated group. The animals received rEPO (400 IU/kg subcutaneously) every other day for one week. Rats were administered saline every other day for one week in control group. Previously, erythropoietin was used for one week to induce the vascular injury in anaesthetized rabbits with the same dose and schedule [23]. On the 8th day of experiment, rats were anaesthetized with urethane and killed.

Tension recording and experimental protocols

Rats were killed by bleeding from vena cava under urethane anesthesia (1.2 g/kg body weight *ip*). Heart and lungs *en bloc* were removed and transferred to ice cold Modified Krebs–Henseleit solution (MKHS) of the following composition (in mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 11.9, KH_2PO_4 1.2 and D-glucose 11.1 (pH 7.4). Both left and right pulmonary arteries were dissected and carefully trimmed of adhering fat and connective tissue and cut into 2–3 mm long rings under a dissecting microscope. The arterial segments were mounted on two stainless steel hooks and suspended in 10 ml organ baths containing MKHS, maintained at 37 °C, and aerated continuously with medical gas (21% O_2 + 5% CO_2 + 71% N_2) mixture. A passive tension of 1.0 g was applied during the equilibration period of 90 min and the bath solution was changed every 15 min [24,25]. Tension was recorded using a high sensitivity isometric force transducer and stored in a computer using LabChart version 5.4.1 software program (Powerlab, AD Instruments, Bella Vista, NSW, Australia) for further analysis.

Tissue viability was examined by recording the contraction to high K^+ (80 mM)-depolarizing solution after the equilibration period. Cumulative concentration–response curves to relaxants were elicited in pulmonary arterial segments pre-contracted with a sub-maximal concentration of phenylephrine (1 μM) to achieve near identical pre-contraction levels. Pretreatment with L-NAME sensitized the pre-contractions to phenylephrine before eliciting relaxation responses to different vasodilators. On the other hand, indomethacin attenuated contractile responses to phenylephrine. Accordingly, an appropriate concentration of phenylephrine, as stated above, was used to achieve matching contraction level of the arterial segments before eliciting relaxations to vasodilators like ACh and SNP [25].

To assess the endothelium-dependent and independent relaxation of pulmonary arterial rings, concentration-dependent response to acetylcholine (ACh; 1 nM–30 μM) and SNP (1 nM–30 μM) was recorded, respectively, in phenylephrine (PE)-pre-contracted pulmonary arterial rings from control and rEPO-treated groups. The preparations were washed with normal MKHS to restore the baseline resting tension. A concentration-dependent (1 nM–10 μM) contractile response to phenylephrine was elicited in the pulmonary arterial rings from both the groups at an increment of 0.5 log unit. To evaluate the influence of rEPO-treatment on EDHF, pulmonary arterial rings from both the groups were pre-incubated with L-NAME plus indomethacin for 30 min before eliciting a concentration-response curve to ACh. A dose-response curve was also drawn to ACh in pulmonary arterial rings in presence of indomethacin and L-NAME separately from control and rEPO-treated rats. Calcium chloride-induced contraction in K^+-depolarized vascular tissue preparations is a standard protocol for the functional assessment of voltage-dependent L-type calcium channels. After equilibration period, pulmonary arterial rings were contracted with K^+-depolarizing solution followed by wash with Ca^{2+}-free MKHS with EGTA solution to remove any extracellular calcium. Then the tissue was incubated with Ca^{2+}-free K^+-depolarizing solution without EGTA for 30 min. Then concentration-dependent contractions to $CaCl_2$ (10 μM–10 mM) were elicited in the control and rEPO-treated group.

Nitrite measurement

In order to evaluate constitutive NOS activity formation of NO was measured as nitrite. Endothelium-intact pulmonary arterial segments of each group were equilibrated in MKHS for 30 min at 37 °C under continuous air bubbling. Approximately equal wet-weight tissues were placed in 2 ml plastic tubes which contained 490 μl of MKHS. 5 μl (10 μM) of 1400 W was added to each tube. These tubes were incubated for 30 min at 37 °C under air bubbling. After this, 5 μl (1 μM) of ACh was added and left for 10 min. Then the tissues were removed and their weights were measured. The remaining solution was used for nitrite estimation as per the manufacturer's instruction. The released nitrite was measured in duplicate by colorimetry (OD at 540 nm), according to the manufacturer's instructions (Quantichrom Nitric Oxide Assay Kit, D2NO-100; BioAssays Systems, USA).

mRNA expression study

Total RNA isolation

Pulmonary artery was isolated in 0.1% diethyl pyrocarbonate-treated and autoclaved phosphate buffer saline (PBS) from control