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Original article

# Erythropoietin has a restorative effect on the contractility of arteries following experimental hypoxia

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ABSTRACT

*Introduction:* The aim of this study was to investigate the effect of erythropoietin on vascular contractility using an in vitro model of hypoxia replicating the hypoxic environment of blood vessels and surrounding adipose tissue in obesity.

*Methods and results:* Pharmacological in vitro studies were carried out on small mesenteric arterial segments from male Wistar rats with and without perivascular fat and endothelium. Contractile responses were investigated by wire myography under normoxia, experimental hypoxia ± erythropoietin and L-NNA. Perivascular fat exerted an anticontractile effect which was lost following the induction of experimental hypoxia. Erythropoietin prevented the loss of the anticontractile capacity when vessels were incubated for one hour before the induction of hypoxia or throughout the period of hypoxia; this was found to be independent of the function of perivascular fat, as fat denuded arteries had a similar reduction in contractility (artery no fat + hypoxia vs. artery no fat + hypoxia + erythropoietin). The mechanism by which erythropoietin was exerting its effect was found to be partially endothelium dependent and associated with an increase of nitric oxide bioavailability as nitric oxide synthase inhibition prevented the effect.

*Conclusions:* Whilst erythropoietin is working downstream from perivascular fat, it is possible that it may be therapeutically useful in obesity when hypoxia and inflammation reduce the normal activity of perivascular fat.



## 1. Introduction

The prevalence of obesity is increasing and is set to reach pandemic levels in many countries.[1] In addition, there is an increased in the incidence of chronic kidney disease (CKD),[2] which is associated with an increased risk of cardiovascular disease.[3,4] Whether obesity itself or its comorbidities, including type 2 diabetes and hypertension, are responsible for the increase risk is not yet clear. Several studies demonstrate an association between obesity and cardiovascular risk, most of which suggest that it is through the influence of other risk factors[5–7]; however, others propose a direct link between obesity and CKD.[8–10] What is apparent, however, is the significance of obesity in this life threatening disease.

Erythropoietin (EPO) is a haematopoietic growth factor used in the treatment of anaemia of a number of etiologies including renal failure.[11] The presence of EPO receptors on cells other than erythroid progenitors, such as endothelial and myocardial cells (reviewed in Ref. 12), indicates a potential biological role beyond its traditional use.[13] A number of studies have shown EPO associated improvement in immunological functions in particular increased insulin sensitivity. EPO related reductions in body mass index (BMI),[14] low density lipoproteins (LDL)[15] and glucose levels[13,16] also indicate EPO signalling may be beneficial in the treatment of obesity and its associated complications including type 2 diabetes. Interestingly, the expression of the adipokine leptin, which correlates with fat mass,[17] is significantly reduced following 3–6 months of EPO treatment.[18]

It is widely accepted that the fat surrounding arteries exerts a beneficial anticontractile effect through the release of adipokines.[19,20] Previously we have shown that in patients with obesity and the metabolic syndrome, the anticontractile capacity of

*Abbreviations:* ACh, acetylcholine; BMI, body mass index; CKD, chronic kidney disease; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; KPSS, high potassium physiological salt solution; LDL, low density lipoproteins; L-NNA, Nω-nitro-L-arginine; NA, noradrenaline; NO, nitric oxide; PSS, physiological salt solution; SEM, standard error of the mean.

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perivascular fat is lost.[21] This is most likely through a hypoxia-induced change in the adipokine profile including a reduction in adiponectin and increase in inflammatory markers such as TNF-α.[22] It has been proposed that obesity is associated with increased adipocyte size[21,23] leading to hypoxia and subsequent inflammation, this in turn abrogates the anticontractile capacity of perivascular fat thus providing a link with hypertension, a common marker for CKD. Despite the potential beneficial effects of EPO in obesity, whether it has the capacity to rescue obesity-induced changes in perivascular fat function on vascular contractility remains to be addressed. The aim of this study was to investigate the effect of EPO using an in vitro model of hypoxia replicating the hypoxic environment of blood vessels and surrounding adipose tissue in obesity.[21,22]

## 2. Methods

### 2.1. Artery preparation

Procedures were performed in accord with Institutional Guidelines and the United Kingdom Animals (Scientific Procedures) Act of 1986. Healthy male Wistar rats (12–15 weeks) were killed by stunning followed by cervical dislocation. The mesenteric bed was immediately removed and placed in ice cold physiological salt solution (PSS) (mM: 119 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.17 $KH_2PO_4$, 1.17 $MgSO_4$, 0.026 EDTA, 1.6 $CaCl_2$ and 5.5 glucose); first order arteries were identified (diameter: 250–300 μm) and part dissected clean of fat, whilst the other part was left with surrounding fat intact.[21,22] Four 2 mm segments of the same arterial branch, two with fat and two without fat, were mounted on a Wire Myograph System (Danish Myo Technology, Denmark). Briefly, arteries were mounted on two 40 μm tungsten wires and fixed to the jaws of the wire myograph. Arteries in PSS were maintained at 37 °C, pH7.4, and bubbled with 95% air/5% $CO_2$. Following a 30 min equilibration period, arterial segments were normalised using a standard procedure, and the internal circumference set to 90% of internal circumference at 100 mmHg as calculated using the Laplace equation, and allowed to equilibrate under stretch for 30 min before initiation of the experimental protocol.[24] Arterial viability was assessed by constriction with high potassium-PSS (60 mM KPSS) and endothelial integrity determined by addition of $1 \times 10^{-5}$ M acetylcholine. Constriction of arteries with 60 mM KPSS was performed after each dose response curve to noradrenaline ($1 \times 10^{-9}$ M–$1 \times 10^{-5}$ M).

### 2.2. Protocols

Baseline dose response curves to noradrenaline ($1 \times 10^{-9}$ M–$1 \times 10^{-5}$ M) were constructed in arteries with and without perivascular fat under normoxia. The contractility of arteries was then investigated following the induction of experimental hypoxia (95% $N_2$/5% $CO_2$ for 2.5 h).[21,22,25] The level of hypoxia was determined using an oxygen probe; levels of oxygen tension of 35 mmHg were achieved within 10 min and were stable throughout hypoxia following which dose response curves to noradrenaline were performed.

To determine whether EPO had protective effects in hypoxia, studies were first carried out to identify the optimum dose of EPO to be used in further experiments. Arteries treated with EPO (10 u/mL) were either preincubated for 1 h before the induction of experimental hypoxia or incubated with EPO throughout the period of hypoxia.

The role of the endothelium in mediating the EPO effects was investigated by endothelial denudation; this was achieved by passing a 40 μm tungsten wire through the lumen of the artery to remove the endothelial layer.[26] Arteries were checked for viability before and after denudation using KPSS and endothelial integrity was determined by relaxation in response to $10^{-5}$ M acetylcholine; arteries were classed as denuded when relaxation to acetylcholine was less than 5%. Experiments in which arteries were incubated with EPO throughout hypoxia were then performed in denuded arteries. To establish whether EPO exerted its effects via nitric oxide bioavailability, arteries under hypoxia in the presence of EPO were incubated with NG-nitro-L-arginine (L-NNA) ($1 \times 10^{-5}$ M).[26]

### 2.3. Chemicals

Chemicals used for PSS and KPSS, noradrenaline, acetylcholine and L-NNA were all sourced from Sigma–Aldrich, Dorset, UK. Erythropoietin was obtained from Jannsen Cilag, UK and prepared in PSS.

### 2.4. Data analysis

Each experiment was performed on arterial segments from different rats. Data were recorded using the PowerLab Lab Chart (version 5.5.6) acquisition program (AD Instruments, Oxford, UK). Data are presented as mean ± SEM. Differences in response to noradrenaline were expressed as a percentage of constriction to 60 mM KPSS in line with previous studies,[19,27] and analysed using a two-way ANOVA with a Bonferroni post hoc test to test differences at each dose response point. $P$ values $<0.05$ were considered significant. Analyses were performed with GraphPad Prism (version 3.00) for Windows (GraphPad Software, California, USA).

## 3. Results

### 3.1. Pharmacological assessment of vessel contractility in rat mesenteric arteries

No significant difference of the contractility of arteries with and without perivascular fat to 60 mM KPSS were observed (data not shown), however the presence of perivascular fat was associated with a significant anticontractile effect in response to noradrenaline (fat vs. no fat: $P < 0.0001$, $n = 14$), This effect was lost following induction of experimental hypoxia (95% $N_2$/5% $CO_2$) for 2.5 h (fat vs. fat + hypoxia: $P = 0.0067$, $n = 14$) (Fig. 1A). EC50 values demonstrated that hypoxia did not significantly change the sensitivity of vessels in the presence of perivascular fat to noradrenaline. Hypoxia did not have a significant effect on arteries without fat (maximum constriction: artery no fat 155.4 ± 6.0% vs. artery no fat hypoxia: 143.7 ± 9.0%, $P = 1745$, $n = 14$).

### 3.2. EPO can part rescue the loss of anticontractile capacity of hypoxia following experimental hypoxia

Incubation with EPO (10 u/mL) throughout the period of hypoxia reduced the loss of the anticontractile capacity perivascular fat ($P = 0.0004$, Hypoxia ($n = 14$) vs. Hypoxia + EPO ($n = 8$)) (Fig. 1B), this was associated with a small, but significant decrease in sensitivity to noradrenaline ($P = 0.048$). 1 h preincubation with EPO (dose) also reduced the effect of hypoxia, although not to the same extent as incubation throughout the period ($P = 0.047$, hypoxia ($n = 14$) vs. hypoxia + EPO preincubation ($n = 5$)) (Fig. 1C) there was an increase in EC50 compared to arteries in hypoxia ($P = 0.043$). Arteries without perivascular fat had no significant increase in contractility following experimental hypoxia ($P = 0.174$).