Journal of Cardiovascular Disease Research 4 (2013) 164–169

Contents lists available at ScienceDirect

Journal of Cardiovascular Disease Research

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

Original article

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

Sarah B Withers [a,*], Neha Passi [a], Alfred S Williams [a], Declan de Freitas [b], Anthony M Heagerty [a]

[a] Cardiovascular Research Group, School of Biomedicine, University of Manchester, Manchester M13 9NT, UK
[b] Manchester Institute of Nephrology and Transplantation, Manchester Royal Infirmary, Manchester M13 9WL, UK

ARTICLE INFO

Article history:
Received 12 March 2013
Accepted 12 August 2013
Available online 16 October 2013

Keywords:
Myograph
Microcirculation
Obesity

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.

* Corresponding author. Tel.: +44 0161 2751229; fax: +44 0161 2751183.
E-mail address: sarah.withers@manchester.ac.uk (S.B. Withers).

0975-3583/$ – see front matter
http://dx.doi.org/10.1016/j.jcdr.2013.08.002

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 1×10^{-5} M acetylcholine. Constriction of arteries with 60 mM KPSS was performed after each dose response curve to noradrenaline (1×10^{-9} M–1×10^{-5} M).

2.2. Protocols

Baseline dose response curves to noradrenaline (1×10^{-9} M–1×10^{-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) (1×10^{-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 ($P = 0.174$).

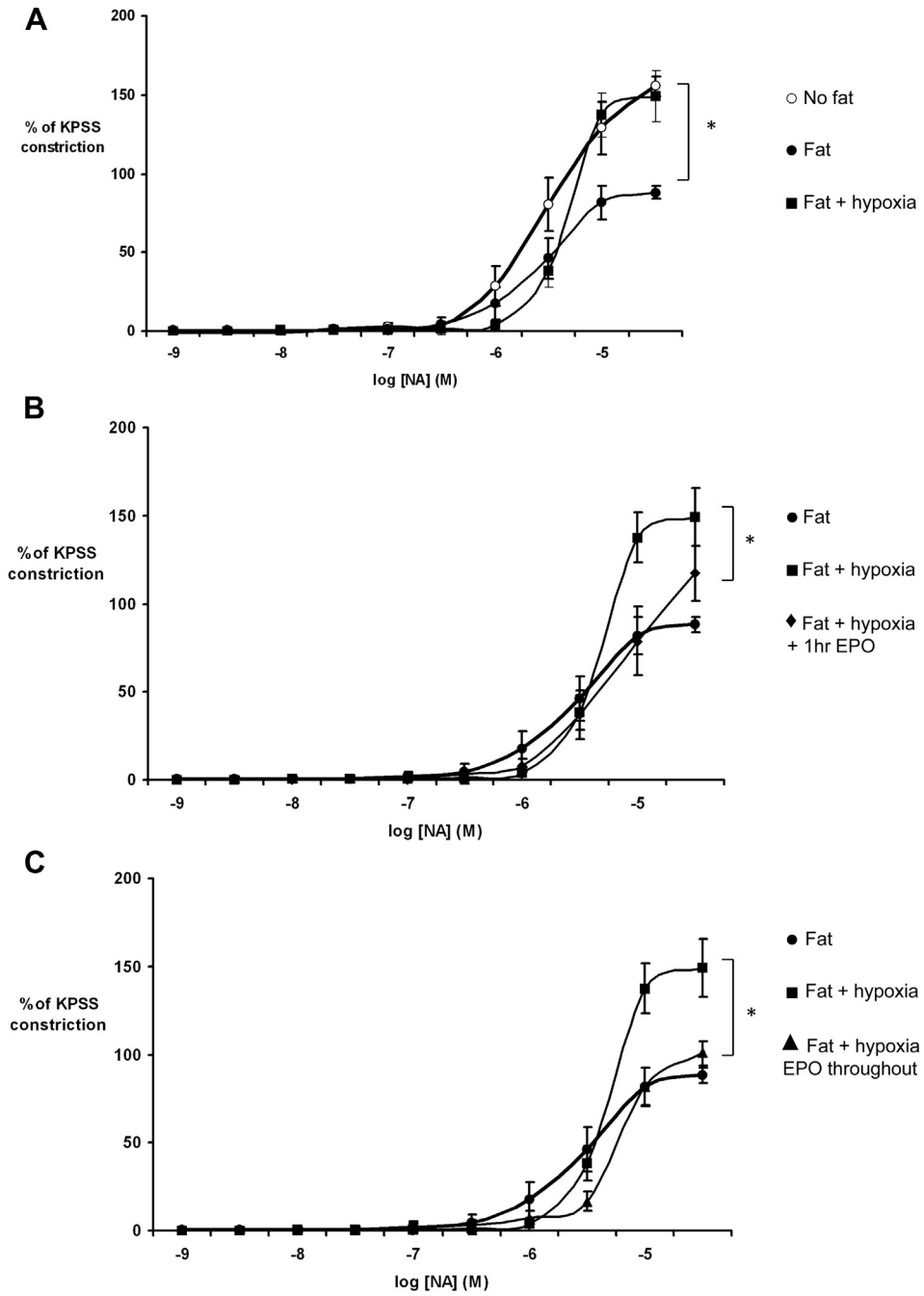

Fig. 1. The effect of hypoxia ± EPO on the contractility of arteries. A. Healthy fat has an anticontractile capacity which is lost following experimental hypoxia (○ = artery with no fat ($n = 14$), ● = artery + fat ($n = 14$), ■ = artery + fat + hypoxia ($n = 14$)). B. 1 h EPO preincubation of arteries with fat partially rescues the loss of anticontractile function during hypoxia (● = artery + fat ($n = 14$), ■ = artery + fat + hypoxia ($n = 14$), ◆ = artery + fat + hypoxia + preincubation EPO ($n = 6$)). C. Incubation with EPO throughout hypoxia restore the anticontractile function of healthy fat (● = artery + fat ($n = 14$), ■ = artery + fat + hypoxia ($n = 14$), ▲ = artery + fat + hypoxia + EPO throughout ($n = 9$)). * = $P < 0.05$.

3.3. *The protective effects of EPO are independent of perivascular fat and part mediated through smooth muscle and endothelium*

Arteries without perivascular fat incubated with EPO throughout hypoxia demonstrated a reduction in contractility ($P = 0.046$, no fat + hypoxia ($n = 14$) vs. no fat + hypoxia + EPO ($n = 8$)) (Fig. 2A). Denudation of arteries with perivascular fat had no protective capacity of EPO compared to arteries with endothelium following hypoxia ($P = 0.4579$, fat + hypoxia ($n = 14$) vs. fat + hypoxia − endothelium ($n = 5$)) (Fig. 2B). Denudation of arteries had no effect on contractility as assessed by constriction in response to KPSS (data not shown).

3.4. L-NNA *inhibits the beneficial effects of EPO*

Incubation of arteries with and without fat with L-NNA during hypoxia + EPO did not exhibit the same beneficial effects of EPO in contractility (Arteries **without** fat: hypoxia + EPO vs. hypoxia + EPO + L-NNA, $P = 0.0156$, $n = 5$; arteries **with** fat hypoxia + EPO vs. hypoxia + EPO + L-NNA, $P < 0.001$, $n = 5$). There

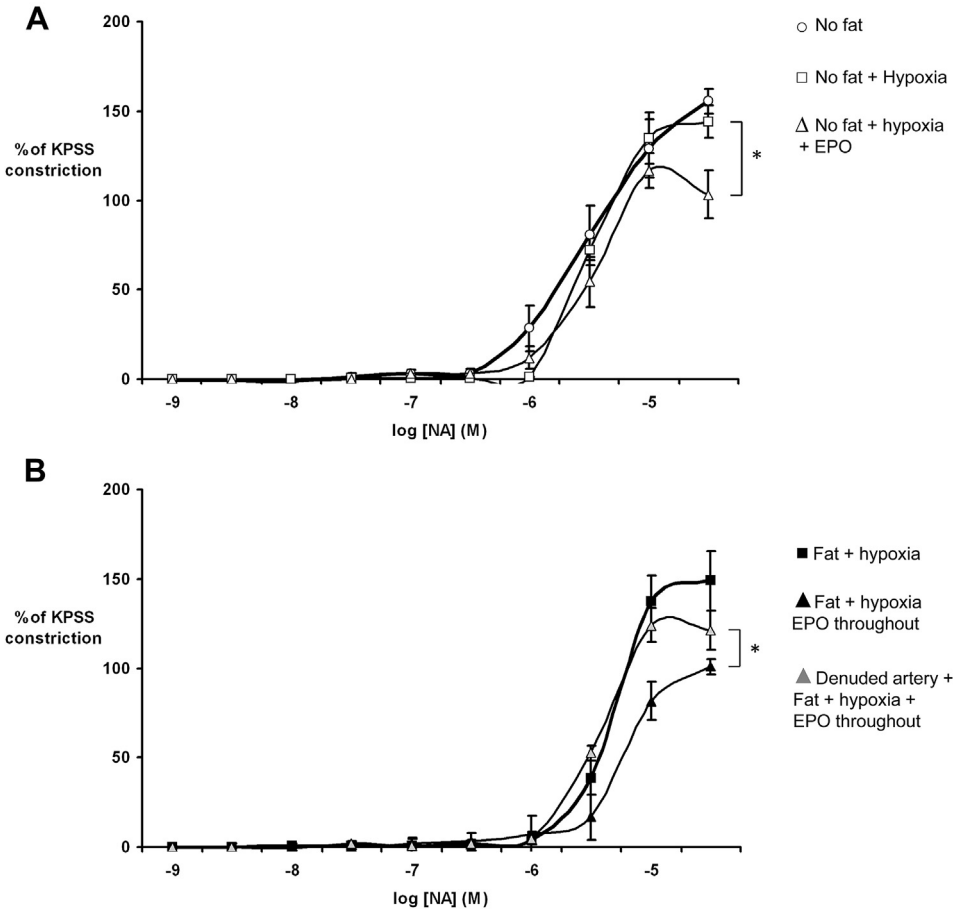

Fig. 2. EPO exerts its effects independent of adipose tissue. A. Arteries without fat show no increase in contractility following hypoxia, however incubation of arteries with EPO throughout hypoxia causes a significant reduction in constriction (○ = artery with no fat ($n = 14$), □ = artery no fat + hypoxia ($n = 14$), Δ = artery no fat + hypoxia + EPO throughout ($n = 9$)). B. The effect of EPO is significantly diminished in the absence of endothelium (■ = artery + fat + hypoxia ($n = 14$), ▲ = artery + fat + hypoxia + EPO throughout ($n = 9$), ▲ = denuded artery + fat + hypoxia + EPO ($n = 5$)). * = $P < 0.05$.

was a significant increase of sensitivity to noradrenaline of arteries in the absence of perivascular fat as determined by the EC50 calculation which was absent in arteries with fat ($P = 0.17$) (Fig. 3A and B).

4. Discussion

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. The important finding in this study is that EPO has a restorative effect on the contractility of arteries following experimental hypoxia. This effect is independent of perivascular fat and involves nitric oxide signalling through endothelium mediated mechanisms. This is perhaps unsurprising as there is no current literature supporting the expression of EPO receptors in adipocytes, indeed Hojman and colleagues demonstrated electrotransfer of EPO into skeletal muscle in a diet-induced model of obesity was associated with reduced fat mass, increased muscle vascularisation and improved metabolic parameters, highlighting that the actions of EPO in skeletal muscle can be beneficial on the adipocyte profile.[28]

Previously we have demonstrated that obesity and the metabolic syndrome contribute to a loss of the anticontractile capacity of perivascular fat through mechanisms involving hypoxia-induced inflammation and subsequent changes in the released adipokine profile.[21] These provide a potential link between obesity and the array of cardiovascular complications including hypertension and CKD.[2,4] EPO is widely used in the treatment of end stage renal disease; however the responsiveness of haemodialysis patients to this drug is impaired when the disease is further complicated by type 2 diabetes.[11,29] In addition, recent studies implicate EPO and its signalling pathways in the regulation of fat mass and glucose metabolism through its effects independent of haematopoiesis.[13,28,30]

EPO receptors are known to be expressed in the endothelium[31] and have been implicated in mediating neuroprotective effects of EPO in cerebral arteries through an enhancement of endothelium-derived hyperpolarizing factor.[32] Therefore we investigated the contribution of the endothelium in mediating the effects of EPO during hypoxia and endothelial denudation. Our results demonstrate that a functional endothelium is necessary in mediating the restorative effects of EPO in our model. This supports previous studies where EPO increased NO bioavailability by upregulation of eNOS in cultured endothelial cells through a direct effect on the endothelium.[33,34] This may go some way to explain EPO-resistance observed in obesity[35]; previously we have demonstrated a loss of endothelial integrity in patients with obesity and the metabolic syndrome through the reduced capacity to dilate to acetylcholine.[21] In addition, Tg6 mice which overexpress EPO have significantly higher levels of NO expression compared with wildtype counterparts[36] which has been suggested to increase insulin sensitivity.[37]

The role of eNOS and nitric oxide bioavailability in mediating the EPO effect was strengthened by our data which demonstrate that L-

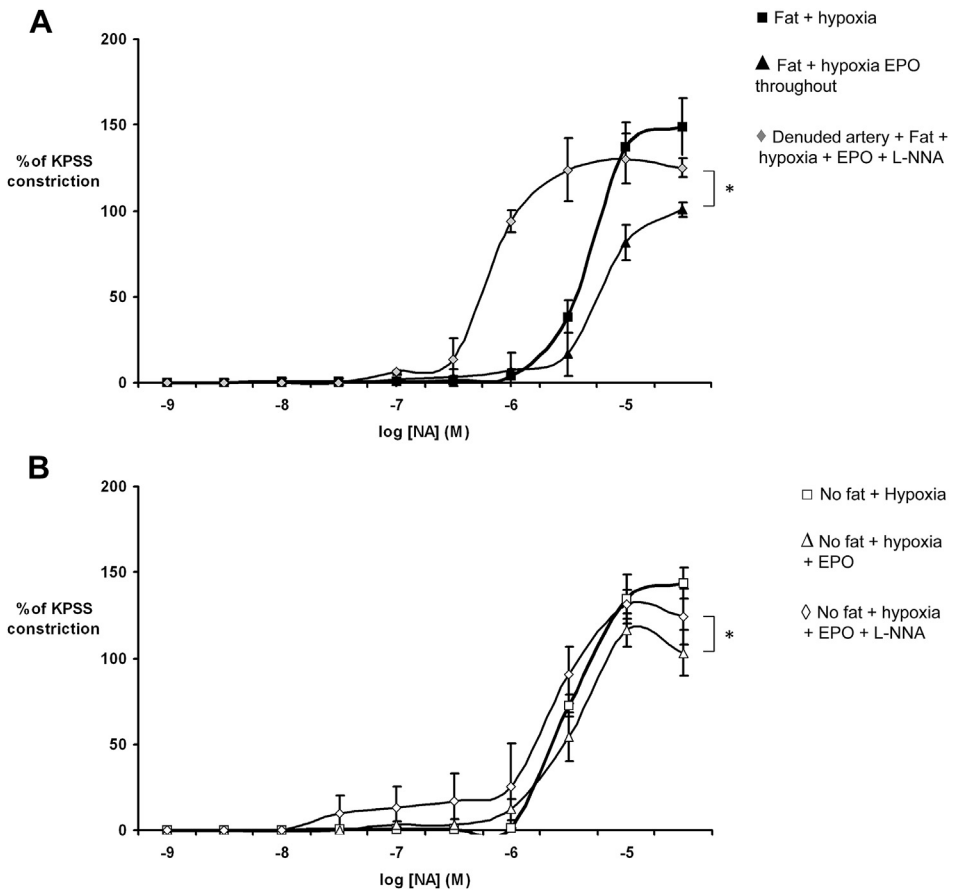

Fig. 3. L-NNA reduces the beneficial effects of EPO suggesting an involvement of NO. A. Arteries incubated with L-NNA during experimental hypoxia + EPO were associated with an increase constriction compared to arteries under hypoxia + EPO (■ = artery fat + hypoxia (n = 14), ▲ = artery fat + hypoxia + EPO throughout (n = 9) ◆ artery + fat + hypoxia + EPO + L-NNA (n = 5)). B. L-NNA reduces the reduction in contractility observed following hypoxia in the presence of EPO (□ = artery no fat + hypoxia (n = 14), Δ = artery no fat + hypoxia + EPO throughout (n = 9), ◇ = artery no fat + hypoxia + EPO throughout + L-NNA (n = 5)). * = $P < 0.05$.

NNA prevented the restorative effect of EPO in hypoxia. This supports previous studies where Beleslin-Cokic and colleagues showed that EPO was able to increase endothelial NO bioavailability by promoting eNOS transcription and also through stimulation of eNOS activity through phosphorylation at serine 1177 during hypoxia.[33] These studies suggest that the main effects of EPO in restoring contractility are through actions on eNOS via smooth muscle or endothelial cells.

Whether our the effects of EPO are due to changes in the adipokine profile in our system has not been investigated; studies have demonstrated that chronic EPO treatment results in increased levels of leptin[28] and resistin,[38] however our investigations are limited to an acute model of contractility and experimental hypoxia. Whether other animal models, such as the obese rat or eNOS deficient mouse, will provide further insight into the protective mechanisms of EPO on vascular contractility remains to be elucidated. However, the use of EPO in the treatment of cardiovascular complications associated with obesity should be approached with caution as Tg6 mice treated overexpressing EPO have a significantly attenuated lifespan due to multiple organ damage.[39] Despite the promising potential of new non-haematological EPO agents for the treatment of ischaemic stroke[40] recently developed, the current phase of agents has not exhibited the same beneficial effects on fat accumulation.[28]

5. Conclusion

In consequence the findings go some way to explain and highlight the complexities of treating CKD in an ever-increasing obese population. This study points towards a further possible therapeutic role of EPO in the treatment of CKD particularly in those patients with obesity and obesity associated complications. Further work is required to determine the exact mechanisms by which EPO mediates its protective effects in the vasculature, although it is clear that it has functions beyond its traditional role.

Conflicts of interest

All authors have none to declare.

Acknowledgements

This work is financially supported by the Wellcome Trust.

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