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The effect of tumour necrosis factor-α and insulin on equine digital blood vessel function *in vitro*

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Abstract

Objective and design: Insulin and inflammatory cytokines may be involved in equine laminitis, which might be associated with digital vascular dysfunction. This study determined the effects of TNF-α and insulin on the endothelial-dependent relaxant responses of equine digital blood vessels and on equine digital vein endothelial cell (EDVEC) cGMP production.

Material: Isolated rings of equine digital arteries (EDAs) and veins (EDVs) were obtained and EDVECs were cultured from horses euthanased at an abattoir.

Methods: The effect of incubation with TNF-α (10ng/ml) and/or insulin (1000μIU/ml) for 1.5 hours or overnight under hyperoxic and hypoxic conditions on carbachol (endothelium-dependent) induced relaxation was assessed. The time course and concentration dependency of the effect of TNF-α and the effect of insulin (1000μIU/ml) on EDVEC cGMP production was determined.

Results: Incubation of EDAs overnight with TNF-α under hypoxic conditions resulted in endothelial-dependent vascular dysfunction. EDVs produced a more variable response. TNF-α increased EDVEC cGMP formation in a time and concentration dependent manner. Insulin had no significant effects.

Conclusions: There is a mismatch between the results obtained from isolated vessel rings and cultured endothelial cells suggesting TNF-α may reduce the biological effect of NO by reducing its bioavailability rather than its formation, leading to endothelial cell dysregulation.

Key Words: Insulin, TNF-α, equine, digital, cGMP, laminitis

Introduction

Laminitis is a common and painful condition of the horse characterised by failure of the attachment of the epidermal cells of the epidermal laminae to the underlying basement membrane of the dermal laminae [1]. Animals at greatest risk of pasture-associated laminitis have a metabolic phenotype including obesity and IR, similar to that seen in human metabolic syndrome (HMS)[2]. Thus the same pathologic mechanisms that underlie the cardiovascular disease associated with HMS, including changes in insulin signalling, inflammatory cytokines and endothelial dysfunction, could contribute to laminitis. Whilst the exact pathogenesis of the disease remains unclear, there is evidence from experimental models of the disease to support roles for inflammation [3-5], vascular and endothelial dysfunction [5-7], insulin resistance [8, 9] and extracellular matrix degradation [10, 11]. However further research is required to fully elucidate the pathways involved and perhaps determine a unifying concept.

In other species, vascular and endothelial dysfunction can be caused by hyperinsulinaemia and/or insulin resistance (IR) [12] and inflammatory mediators [13]. Normally, insulin activates both the phosphatidylinositol (PI3) kinase pathway resulting in stimulation of endothelial nitric oxide synthase (eNOS) and hence vasodilation and the mitogen activated protein (MAP) kinase pathway resulting in enhanced production of endothelin-1 (ET-1) and hence vasoconstriction [14]. IR is characterised by specific impairment of the PI3-kinase-dependent signaling pathway without affecting the MAP-kinase pathway, thus nitric oxide (NO) production is decreased whilst ET-1 production remains unchanged resulting in vasoconstriction [15]. Tumour necrosis factor-α (TNF-α) is a pro-inflammatory cytokine secreted by leukocytes and various other cells, including the non fat cells in adipose tissue of obese individuals, which plays an important role in HMS [13]. This cytokine is implicated in

vascular and endothelial dysfunction, mainly through promotion of redox signalling to inhibit flux through the PI-3 kinase pathway distal to the insulin receptor or via activation of serine kinases [16, 17].

Isolated equine digital blood vessels have been used extensively to study the receptors involved in vasoconstriction and vasodilation [18, 19] and the effects of various drugs [20], mediators [21], cooling [22] and hypoxia [23]. The effect of inflammation on equine digital blood vessels has been mostly investigated previously by exposing the vessels to endotoxin [24-26]. The effect of tumour necrosis factor-α (100pg/ml) on equine digital arterial function has been evaluated once previously [27]. The effects of insulin have also been investigated previously. In one study, equine digital arteries and veins were exposed to extremely high concentrations of insulin (10μmol/l or >1,000,000μIU/ml) for 30 mins, contracted with phenylephrine and then a second equally high dose of insulin (10μmol/l) was added [28]. In separate study, the effect of insulin (1000μIU/ml) on the responses of equine small laminar veins to the vasoconstrictors noradrenaline, phenylephrine, ET-1 and 5-hydroxytryptamine (5-HT) was evaluated [29].

The aim of the present study was to determine whether inflammation induced by TNF-α caused impaired vasodilation in equine digital blood vessels, through decreased endothelial nitric oxide (NO) production. This was achieved by investigating the effects of TNF-α on the endothelium-dependent relaxant response of isolated rings of equine digital arteries (EDAs) and veins (EDVs) under hypoxic and hyperoxic conditions and on cGMP (marker of NO production) mediator production by cultured equine digital vein endothelial cells (EDVECs). Furthermore, the effect of insulin was similarly examined to investigate whether high insulin concentrations cause further endothelial dysfunction and exacerbate the effect of TNF-α.

Materials and Methods

Isolated rings of equine digital arteries (EDAs) and veins (EDVs)

The hind limbs of healthy, mixed breed horses, euthanased at an abattoir for purposes other than research, were removed within 10 minutes of death. EDAs were cannulated at the level of the metatarsal-phalangeal joint and flushed with 150 ml cold, oxygenated modified Krebs-Henseleit (Krebs) solution (composition [mM]: $CaCl_2$ 1.27, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, NaCl 118, KH_2PO_4 1.19, KCl 4.57, glucose 5.55). The legs were then transported to the laboratory. The skin over the lateral aspect of the pastern was removed to reveal the digital artery and vein which were carefully dissected free from surrounding tissues, cleaned of connective tissue and cut into 4-5mm long rings. Vessel rings were stored in pre-oxygenated Krebs at 4°C overnight. Only one EDA and one EDV from each horse were harvested and each was only used in a single study.

Tension Recording in Isolated EDAs and EDVs

Twelve vessel rings could be studied simultaneously in a single study. Vessel rings from the same horse maintained under different incubation conditions were always evaluated during the same experiment. The vessel rings were suspended between two parallel stainless steel wires bathed in 10 ml Krebs solution in an organ bath at 30°C bubbled with either 95% O_2 and 5% CO_2 (designated hyperoxia) or 95% N_2 and 5% CO_2 (designated hypoxia). These gas mixtures provide standard conditions for isolated blood vessel studies [30], as originally described in the work leading to the discovery of the role of nitric oxide in endothelium-dependent relaxation [31]. One of the wires was fixed and the other was connected to an isometric force transducer (HSE force transducer type K30, Linton Instrumentation Ltd, Norfolk, UK). The output of the transducer was fed via an amplifier (HSE type 301; Linton

Instrumentation Ltd) to a data acquisition system (PowerLab; AD Instruments, Oxfordshire, UK). Once mounted, EDA and EDV rings were stretched to 3 g and 2g resting tension, respectively, and allowed to equilibrate for 1 h. Previous studies had shown that this protocol was optimal for the measurement of vasoconstrictor and vasorelaxant responses in this vessel type [18, 32, 33].

The viability of each vessel segment was tested by exchanging the Krebs solution for one in which the sodium chloride had been replaced with potassium chloride to produce a depolarising Krebs solution (DKS; 118 mM KCl). This evaluates the activation of smooth muscle contractile elements to an influx of extracellular Ca^{2+}, without specific receptor activation. Once the tension had increased to reach a plateau, the vessels were washed three times with Krebs solution and allowed to relax to baseline tension. As is standard procedure, vessel rings that failed to increase their tension by >50% of the baseline tension in response to DKS were considered non viable and were discarded [30, 34].

Phenylephrine (PHE, 10^{-6} M; Sigma-Aldrich Company Ltd, Dorset, UK) was then added to the Krebs solution to constrict the vascular smooth muscle. This concentration has previously been shown to produce approximately 50% of maximum tension in equine digital arteries and veins [35, 36]. Once a plateau was reached, carbachol (CCh, 10^{-6} M; Sigma-Aldrich) was added to produce vasodilation. CCh is a cholinergic (muscarinic receptor) agonist that causes endothelial nitric oxide-dependent vasodilation and is thus used to assess the integrity of the endothelium. If the endothelium is damaged during vessel preparation, relaxation to CCh does not occur. The percentage relaxation of PHE-induced tone was calculated, and the vessels were discarded unless a minimum of 50% relaxation to CCh occurred. The vessels were then washed three times with Krebs solution and allowed to relax to baseline tension.

The Effect of TNF-α and Insulin on Relaxant Responses in EDAs and EDVs under Hyperoxic and Hypoxic Conditions

After assessing viability and allowing equilibration back to baseline tension, EDAs (n=6 for each experiment) and EDVs (n=6 for each experiment) were incubated with recombinant equine TNF-α (10ng/ml) and/or insulin (1000μIU/ml) for either 1½ hours or overnight (at 37˚C) and bubbled with either 95% oxygen and 5% carbon dioxide (designated hyperoxia) or 95% nitrogen and 5% carbon dioxide (designated hypoxia). These gas mixtures provide standard conditions for isolated blood vessel studies [30], as originally described in the work leading to the discovery of the role of nitric oxide in endothelium-dependent relaxation [31]. PHE (10^{-6}M) was then added to the Krebs to constrict the vessels and, once a plateau of contraction was reached, the carbachol (endothelium-dependent) induced relaxation was again assessed by serially adding increasing concentrations to generate a cumulative concentration response curve.

Equine Digital Vein Endothelial Cell Culture

Equine digital vein endothelial cells (EDVEC) were cultured as previously described [37] from the digits of horses euthanased at an abattoir for purposes other than research. Briefly, as soon as possible post mortem, the digits were flushed to remove the blood with sterile phosphate buffered solution (PBS; 150 ml) by cannulating the medial and lateral digital veins 3 to 4 cm above the coronary band. The medial and lateral digital arteries were then ligated, the digit was infused from the venous side with type II collagenase[a] (20ml; 1mg/ml; prewarmed at 37˚C) and the limb was incubated in a water bath for 30 min at 37ºC. The endothelial cells were then flushed out and collected using sterile PBS before being centrifuged (300 x g for 10min). The supernatant was removed and the cells resuspended in culture medium (Dulbecco's modified Eagles medium[b] containing 10% foetal calf serum,

10% newborn calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin) and transferred to a 75cm^2 flask for incubation at 37°C in 5% CO_2 and 95% air. After 24 hours, erythrocyte contamination was removed with warm sterile PBS and fresh culture medium added. Once 90% confluency was achieved, characterized by the typical cobblestone morphology and positive immunostaining for von Willebrand's factor, the cells were lifted with trypsin and EDTA solution (1 mg/ml and 0.25 mg/ml, respectively), resuspended in culture medium, transferred evenly to 24 well plates and incubated at 37°C for 48 hours to allow the cells to adhere and become confluent.

Effect of TNF-α and Insulin on Mediator Production by EDVEC

Confluent EDVEC monolayers were made quiescent in serum-free medium for 3 hours and pre-incubated with the phosphodiesterase inhibitor, 3-Isobutyl-1-methyl-2,6(1H,3H)-putinedione-Methyl-3-isobutylxzanthine[c] (IBMX, 1mM) for 30 min at 37°C to inhibit breakdown of cyclic GMP (cGMP). EDVEC were then incubated with DMEM containing 1% bovine serum albumin in the presence or absence of recombinant equine TNF-α (10ng/ml) and/or insulin (1000 μIU/ml). The culture medium was sampled after 0, 6, 18 and 24 hours incubation. EDVEC were also incubated for 18 hours with varying concentrations of TNF-α alone (0 – 1000 ng/ml).

Protein and Mediator Determination

Cyclic GMP concentrations were measured as an index of endothelial NO production using a commercial enzyme immunoassay system (Biotrak cGMP EIA; Amersham Pharmacia Biotech., Amersham, Buckinghamshire, UK) according to the manufacturer's instructions (Protocol 2) as previously described [38, 39]. Mediator concentrations were expressed per μg

of protein present in the well, measured following cell lysis using a BCA protein assay kit (Pierce Bio Science Ltd., Tattenhall, Cheshire, UK).

Curve fitting and Statistical Analysis

Statistical analyses were carried out using computer software (Graphpad Prism, version 6.0; GraphPad Inc.). Concentration-response curve data were fitted to a single site sigmoidal response curve model (variable slope) to give values for maximum relaxation (Emax) and EC_{50} values. Normality of the distribution of the data was assessed using the Kolmogorov–Smirnov test. Values represent mean ± sem from n=6-12 (see Figure legends), referring to the number of individual animals from which cells or blood vessels were derived. EC_{50} values were presented as the geometric mean with 95% confidence intervals. The effect of TNF-α and insulin on the maximum relaxant responses of EDAs and EVDs under hyperoxic and hypoxic conditions was evaluated using a paired Student's t-test or one way analysis of variance with Dunnett's *post hoc* test. The effect of TNF-α and insulin on EDVEC mediator production was analysed using a linear mixed effect model. Significance was accepted at $p<0.05$.

Results

There was no significant effect of incubation with 10 ng/ml TNF-α and/or 1000 μIU/ml insulin for 1½ hours, under hyperoxic or hypoxic conditions, on carbachol (endothelium-dependent) induced relaxation of isolated rings of equine digital arteries (EDAs; Figure 1) or equine digital veins (EDVs; Figure 2). The curve fitting parameters calculated from these experiments are presented in Table 1. However, despite the short-term treatment having no appreciable effect, incubation with the same concentration of TNF-α overnight at 37°C under hypoxic conditions significantly ($p<0.05$) decreased the carbachol (endothelium-dependent)

induced relaxation of EDAs (Figure 3). There was no effect of insulin under hyperoxic or hypoxic conditions, even at high concentrations (Figure 4). Finally, incubation with high concentrations of both TNF-α and insulin overnight under hypoxic conditions significantly reduced carbachol (endothelium-dependent) induced relaxation of EDVs; however, again this effect was not observed under hyperoxic conditions (Figure 5).

TNF-α (10ng/ml) stimulated significant increases of 4.0$\pm$2.5 and 2.9$\pm$3.1 fold ($p<0.05$) in EDVEC production of cGMP above basal levels after 18 and 24 hours, respectively (Figure 6). This stimulation was TNF-α concentration dependent (Figure 6). Insulin (1000 μIU/ml) had no significant effect alone or when added in combination with TNF-α (Figure 6).

Discussion

Many studies have hypothesised that reduced lamellar blood flow is a key event in the pathogenesis of laminitis [25, 40-42], with ischaemia and subsequent reperfusion resulting in lamellar damage. However, the results of studies of laminar and digital blood flow using *in vivo* techniques have been conflicting, with increases in blood flow in some studies [43-45] and decreases in others [40, 46-48]. This apparent disagreement may be a consequence of a lack of sensitivity and specificity of the methods used to detect changes in laminar blood flow. *In vitro* studies make it possible to investigate the direct effects of individual factors on the function of specific equine digital blood vessels.

Animals at greatest risk of pasture-associated laminitis have a metabolic phenotype that includes obesity and IR [2]. The prolonged experimental infusion of insulin to induce hyperinsulinaemia resulted in the clinical and histological development of laminitis [49, 50]. Hyperinsulinaemia and IR may predispose to laminitis via vascular function disturbances. Obesity may predispose to laminitis through IR or inflammation as adipocytes are endocrinologically active producing a variety of mediators, some of which antagonise insulin resulting in IR and some of which are pro-inflammatory [51]. TNF-α is one such pro-inflammatory cytokine secreted mainly by the non fat cell within adipose tissue [52], which is associated with endothelial dysfunction [13]. Within a herd of inbred obese ponies, laminitis prone animals had significantly higher plasma TNF-α concentrations compared to normal ponies thus associating laminitis predisposition with increased circulating inflammatory cytokines [53]. Plasma TNF-α concentration is also an independent risk factor for increased IR in horses [54].

TNF-α appears to have variable effects on vascular function in other species. Intracranial injection of TNF-α *in vivo* constricted pial arterioles and reduced cerebral blood flow [55-57]; TNF-α induced pro-contractile effects in coronary arteries [58, 59]; and TNF-α impaired acetylcholine-induced vasodilation of mouse thoracic aorta [60]. In contrast, TNF-α dilated cerebral, mesenteric and cremaster muscle arterioles and relaxed endothelium-denuded aortas [61-66]. In bronchial arteries, TNF-α initially induced dilation, followed by constriction 2 hrs later [67]. Finally, TNF-α (1mM) had no effect on bradykinin-mediated endothelium-dependent relaxant of human omental arteries [68] and TNF-α (10ng/ml) had no effect on the diameter of isolated resistance arteries from the rat cremaster muscle [69]. The effect of TNF-α on equine digital vascular function has only been investigated once previously [27]. Incubation with TNF-α (0.1 or 1ng/ml) for 10 mins significantly decreased the endothelial-dependent relaxation of equine palmar digital arteries to the muscarinic receptor agonist acetylcholine and significantly increased the maximal contraction to noradrenaline [27]. Reported plasma TNF-α concentrations in normal horses range from 1ng/ml [70] to 14ng/ml [71]. In the present study incubation of equine digital arteries for longer (either 90 mins or overnight) with a higher (10ng/ml), but still clinically relevant concentration of TNF-α under hyperoxic conditions had no effect on endothelial-dependent relaxation. However, overnight incubation under hypoxic conditions resulted in endothelial-dependent vascular dysfunction. Equine digital veins showed greater variability in the degree of vasorelaxation but incubation with TNF-α and insulin overnight under hypoxic conditions significantly inhibited vasodilation. The effect of hypoxia on equine digital vascular function has only been investigated once previously; short term (3 hrs) hypoxia enhanced the contractile responses of equine digital arteries [23]. To the authors' knowledge, the effect of TNF-α on vascular function under hypoxic conditions has not been previously evaluated in any species.

Previous studies have produced variable results with respect to the effects of insulin on equine blood vessel function. Infusion of insulin into healthy Standardbreds resulted in mean serum insulin concentrations of approximately 1000μIU/ml and the hoof wall surface temperature (HMST) was higher and less variable once hyperinsulinaemia was established, when compared to control horses, suggestive of insulin-mediated increased digital perfusion [50]. In contrast, *in vitro,* following induction of IR using supra physiologic concentrations of insulin (>1,600,000μIU/ml), instead of the normal relaxation responses, a further equally high dose of insulin resulted in contraction of isolated rings of equine palmar digital arteries [28] and short-term hyperinsulinaemia (142 μIU/ml) led to increased vascular resistance within the isolated equine digit in a model of extracorporeal perfusion [72]. Serum insulin concentrations of up to approximately 600μIU/ml have been recorded in previously laminitic ponies[73]; thus in the present study an insulin concentration of 600μIU/ml was used. This high but still clinically relevant concentration of insulin alone had no effect on endothelium-dependent relaxation.

The combined effect of insulin and TNF-α has been previously evaluated only in humans and rats. Infusion of TNF-α (17ng/min) inhibited endothelium-dependent vasodilation of the brachial artery in humans and the inhibitory effect on vasodilator function was greater with concurrent insulin infusion (0.05mg/kg/min) [74]. Alone neither insulin nor TNF-α had an effect, but TNF-α inhibited the vasodilator but not vasoconstrictor effects of insulin in isolated cremaster muscle resistance arteries, resulting in insulin-mediated vasoconstriction in the presence of TNF-α [69]. In the present study, a high but clinically relevant concentration of insulin did not alter the effects of TNF-α on equine digital vascular function.

The endothelium normally produces vasodilator mediators including nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarising factor (EDHF) [75] and the potent vasoconstrictor endothelin-1 (ET-1) [76]. Endothelial dysfunction results in an imbalance between the production of vasoconstrictory and vasodilatory mediators predominantly due to reduced endothelium-dependent NO bioavailability [77] and hence favouring vasoconstriction. Endothelial dysfunction may occur as a result of insulin resistance and inflammation [14].

In other species there is a large amount of evidence to suggest that TNF-α is associated with endothelial dysfunction [78-81]. TNF-α (10ng/ml) impaired NO production by bovine aortic endothelial cells in a time and dose-dependent manner due to a reduction in eNOS expression [82-86]. TNF-α (10ng/ml) depressed human aortic endothelial cell NO production via the coordinate down regulation of both eNOS expression and argininosuccinate synthase [87], the enzyme which produces arginine, a key substrate for NO synthase. In the present study cGMP was measured as it is a marker of NO biological activity [88]. TNF-α increased cGMP formation by cultured equine digital endothelial cells in a time and concentration dependent manner.

Insulin increased human umbilical endothelial cell NO production in a dose dependent manner with an ED_{50} of 500nM [89] and NOS activation in a dose and time dependent manner, an insulin concentration of 600nM was required to activate eNOS and the effect was maximal after 30 mins [90]. Insulin (500nmol/L) also increased eNOS activation in bovine aortic endothelial cells [91]. In the present study, high clinically relevant concentrations of insulin were used. These concentrations of insulin were 5-fold lower than these previous studies and had no effect on endothelial cGMP production over a much longer time period.

A limitation of the current study was that it used large digital vessels. Whilst similar vessels have been used in several other studies attempting to further elucidate the pathogenesis of equine laminitis [18, 41], it must be acknowledged that there are differences in the vascular responses of large conductance or capacitance vessels and small resistance vessels [41]. Thus caution is advised in extrapolating results of the current study to other vessel types within the equine digit.

In conclusion, incubation of equine digital arteries overnight with high clinically relevant concentrations of the inflammatory cytokine TNF-α under hypoxic conditions resulted in endothelial-dependent vascular dysfunction. High clinically relevant concentrations of insulin did not have a similar effect. Equine digital veins showed greater variability in the degree of vasorelaxation but incubation with TNF-α and insulin overnight under hypoxic conditions also significantly inhibited vasodilation. By contrast, clinically relevant concentrations of TNF-α increased cGMP formation by cultured equine digital endothelial cells. Thus, there is a mismatch between results obtained in isolated endothelial cells and intact blood vessels suggesting that TNF-α may reduce the biological effect of NO by reducing its bioavailability rather than its formation, leading to endothelial cell dysregulation. However, the mechanisms involved require further investigation.

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Table 1. Concentration-response curve parameters for the effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital arteries and veins, incubated under hyperoxic or hypoxic conditions for 1½ hours. For graphs see Figs 1 and 2. CL—confidence limits. No significant differences were observed between treatment and control groups (oneway ANOVA for arteries and paired *t*-tests for veins).

Treatment	**EC50 (M) Geometric mean (95% CL)**		**Emax (% relaxation of PHE-induced tone) mean ± sem**	
	arteries	**veins**	**arteries**	**veins**
Hypoxia:				
Control	6.17 (2.48-9.86) x 10^{-8}	33.2 (2.07-4.57) x 10^{-8}	79.82 ± 4.30	44.58 ± 7.58
TNF-α (10ng/ml)	4.96 (1.61-8.31) x 10^{-8}		74.40 ± 6.28	
insulin (1000μIU/ml)	4.47 (3.02-5.94) x 10^{-8}		70.95 ± 6.46	
TNF-α + insulin	5.96 (1.43-10.50) x 10^{-8}	27.6 (2.14-3.38) x 10^{-8}	71.93 ± 5.42	50.94 ± 9.22
Hyperoxia:				
Control	10.04 (0.51-1.57) x 10^{-8}	90.4 (0.15-19.50) x 10^{-8}	76.61 ± 4.99	51.50 ± 7.53
TNF-α (10ng/ml)	13.8 (0.73-2.03) x 10^{-8}		74.23 ± 3.80	
insulin (1000μIU/ml)	9.91 (5.63-14.20) x 10^{-8}		69.38 ± 4.09	
TNF-α + insulin	10.4 (0.61-1.42) x 10^{-8}	44.5(3.13-5.77) x 10^{-8}	71.09 ± 5.02	55.04 ± 6.43

Table 2. Concentration-response curve parameters for the effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital arteries and veins, incubated overnight at 37°C under hypoxic or hyperoxic conditions. For graphs, see Figs 3,4 and 5.
* and ** indicate significant differences between treatment and control groups ($p<0.05$ and $p<0.01$, respectively; paired t-test).

Treatment	**EC_{50} (M) Geometric mean (95% CL)**		**Emax (% relaxation of PHE-induced tone) mean ± sem**	
	arteries	**veins**	**arteries**	**veins**
Hypoxia:				
Control	9.54 (3.42-15.65) x 10^{-8}		68.80 ± 4.42	
TNF-α (10ng/ml)	7.38 (3.92-10.83) x 10^{-8}		47.81 ± 6.74**	
Control	14.1 (-2.12-3.03) x 10^{-8}		55.79 ± 8.93	
Insulin	11.6 (0.35-1.98) x 10^{-8}		56.99 ± 9.56	
Control	5.75 (2.29-9.21) x 10^{-8}	74.1 (1.29-13.53) x 10^{-8}	68.43 ±6.87	33.93 ± 7.48
TNF-α + insulin	14.5 (0.68-2.22) x 10^{-8}	33.2 (2.73-3.91) x 10^{-8}	44.77 ± 10.05 *	21.32 ± 2.11 *
Hyperoxia:				
Control	13.3 (0.82-1.84) x 10^{-8}	46.9 (-0.26-9.64) x 10^{-8}	61.20 ± 2.80	44.66 ± 8.71
TNF-α (10ng/ml) + insulin (1000μIU/ml)	15.1 (0.85-2.18) x 10^{-8}	120.0 (-0.36-2.76) x 10^{-8}	55.23 ± 5.10	39.89 ± 8.94

Figure Legends

Figure 1: The effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital arteries incubated under hyperoxic (A) or hypoxic (B) conditions for 1½ hours. Each point represents mean ±sem from 6 separate experiments. No significant differences were observed between treatment and control groups (see Table 1 for curve fitting parameters).

Figure 2: The effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital veins incubated under hyperoxic (A) or hypoxic (B) conditions for 1½ hours. Each point represents mean ±sem from 6 (A) or 10 (B) separate experiments. No significant differences were observed between treatment and control groups (see Table 1 for curve fitting parameters).

Figure 3: The effect of TNF-α (10ng/ml) on carbachol-induced relaxation of equine digital arteries incubated overnight under hypoxic conditions. Each point represents mean ±sem from 12 separate experiments. The maximum relaxation was significantly reduced with TNF-α treatment (see Table 2 for curve fitting parameters).

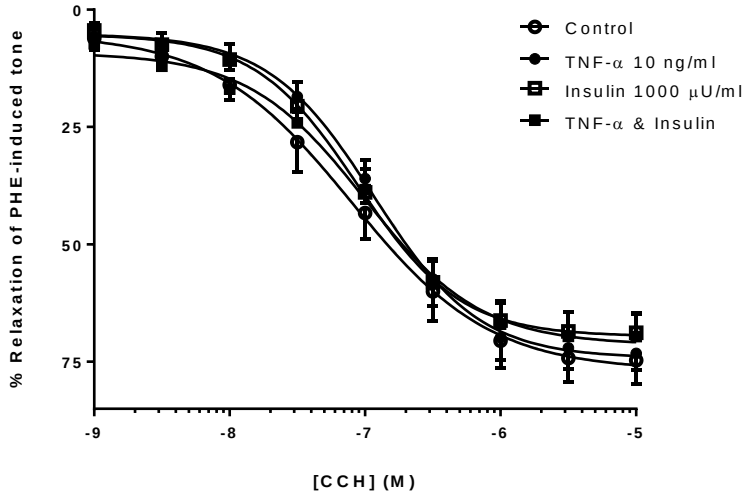
Figure 4: The effect of (A) insulin (1000μIU/ml) and (B) TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital arteries incubated overnight at 37°C under hypoxic conditions. C: The effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital arteries incubated overnight at 37°C under hyperoxic conditions. Each point represents mean ±sem from 6 separate experiments. Under hypoxic conditions, the maximum relaxation was significantly reduced with TNF-α + insulin treatment (Panel B; see Table 2 for curve fitting parameters).

Figure 5: The effect of TNF-α (10ng/ml) and insulin (1000μIU/ml) on carbachol-induced relaxation of equine digital veins incubated overnight at 37ºC under hyperoxic (A) or hypoxic (B) conditions. Each point represents mean ±sem from 6 (A) or 7 (B) separate experiments. Under hypoxic conditions, the maximum relaxation was significantly reduced with TNF-α + insulin treatment (Panel B; see Table 2 for curve fitting parameters).

Figure 6: The (A) time course and (B) concentration dependency of the effect of TNF-α on cGMP production by equine digital vein endothelial cells. Data were analysed using a linear mixed effect model. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figures

Figure 1



B

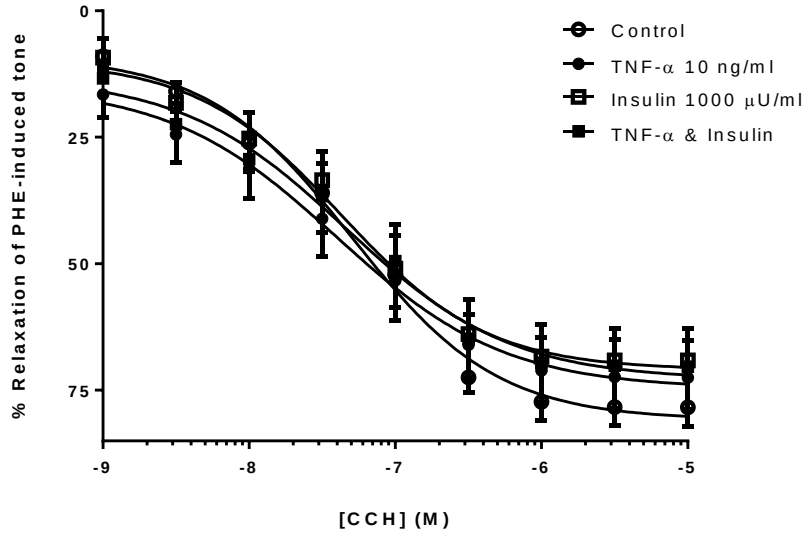
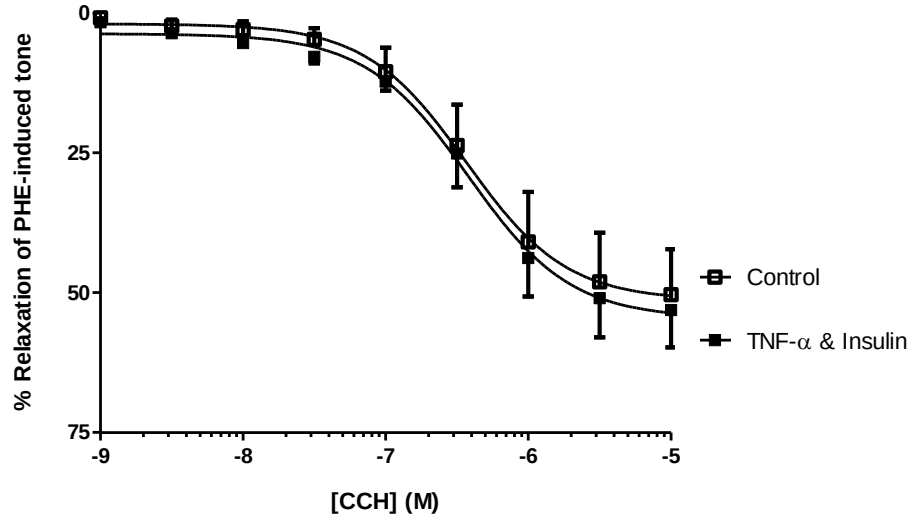


Figure 2

A


% Relaxation of PHE-induced tone
0
25
50
75
-9
-8
-7
-6
-5
[CCH] (M)
Control
TNF-α & Insulin

B

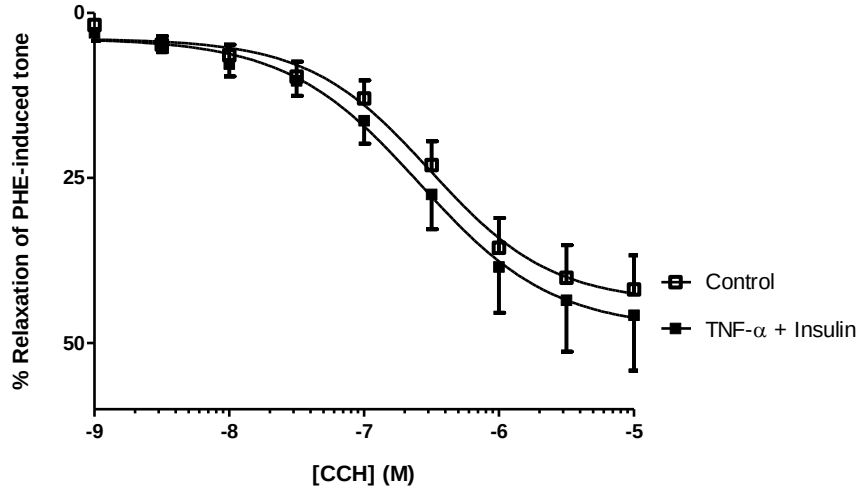

% Relaxation of PHE-induced tone
0
25
50
-9
-8
-7
-6
-5
[CCH] (M)
Control
TNF-α + Insulin

Figure 3

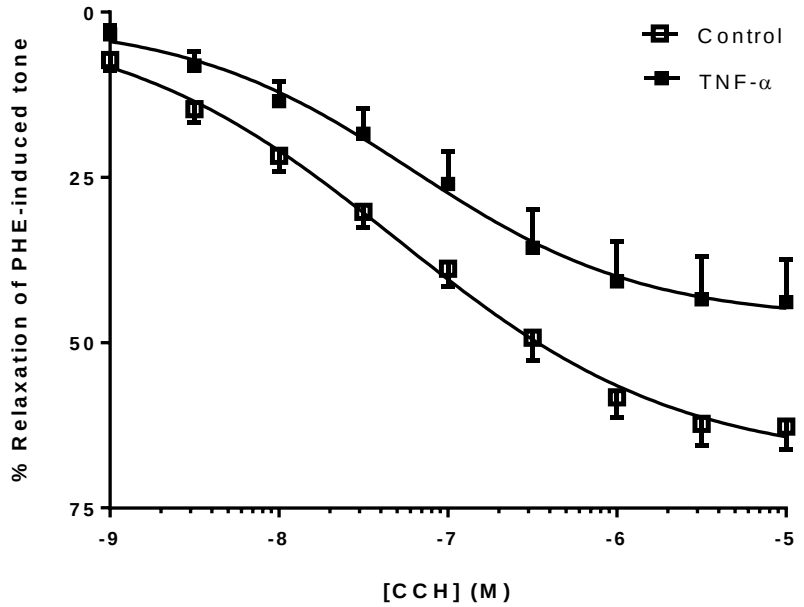
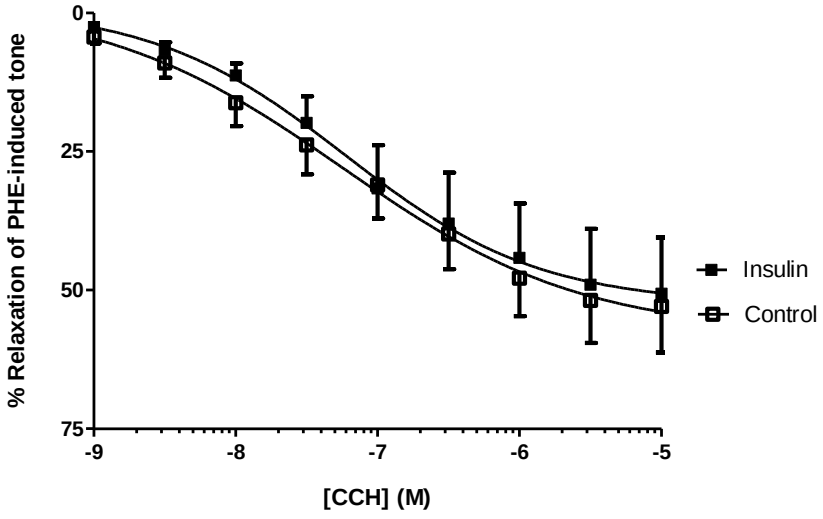
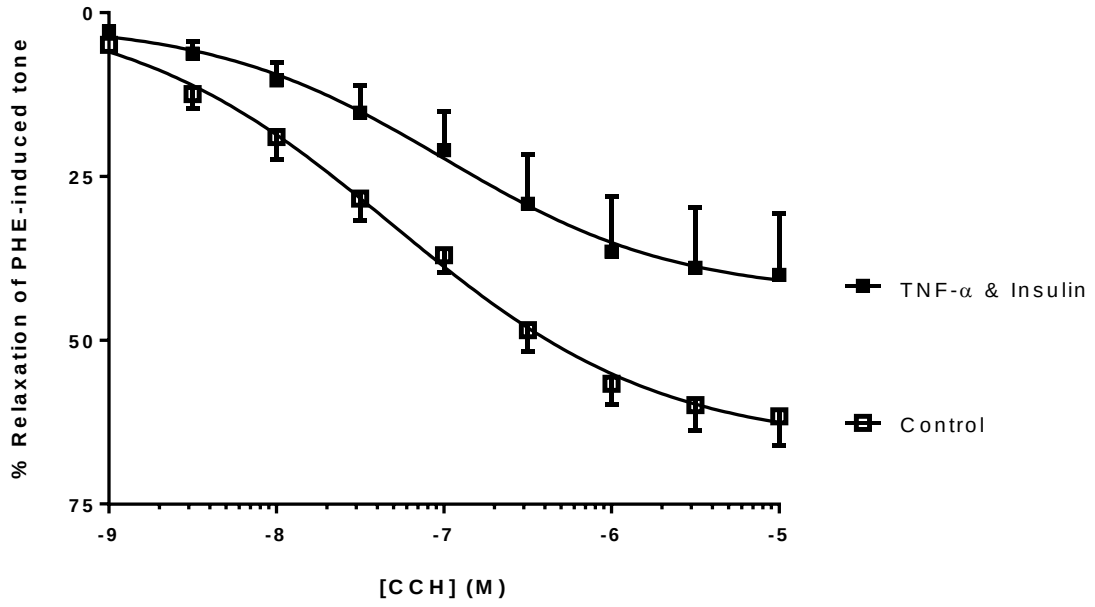


Figure 4

A



B


% Relaxation of PHE-induced tone
0
25
50
75
-9
-8
-7
-6
-5
[CCH] (M)
TNF-α & Insulin
Control

C

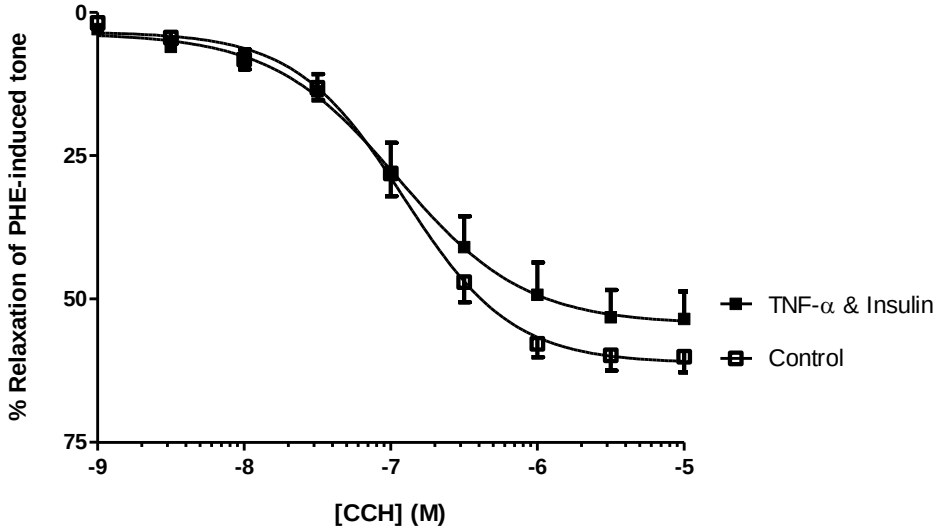
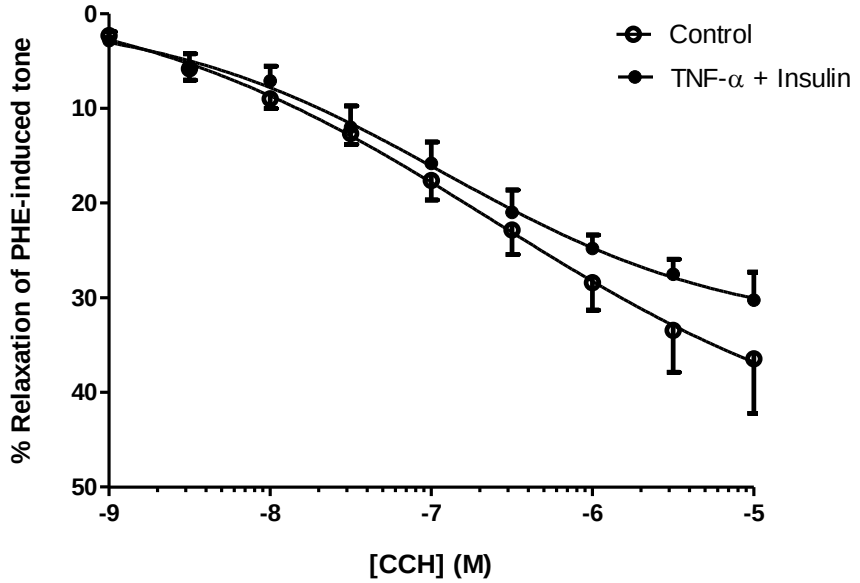

% Relaxation of PHE-induced tone
0
25
50
75
-9
-8
-7
-6
-5
[CCH] (M)
TNF-α & Insulin
Control

Figure 5

A


0
10
20
30
40
50
% Relaxation of PHE-induced tone
-9
-8
-7
-6
-5
[CCH] (M)
Control
TNF-α + Insulin

B

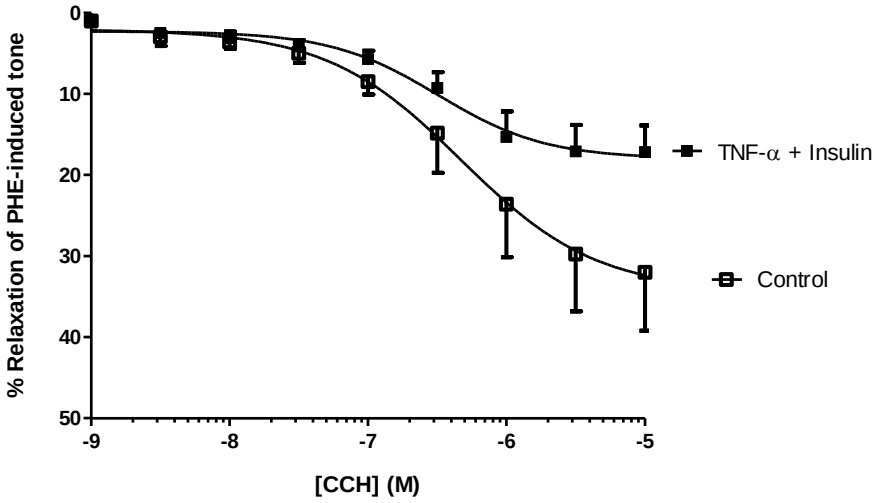
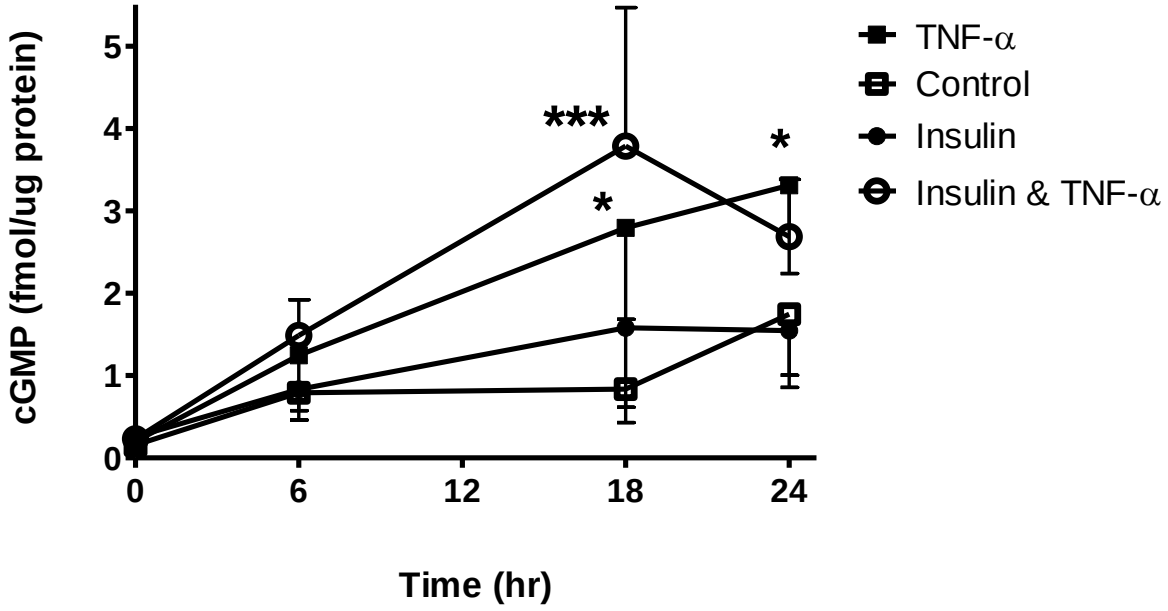
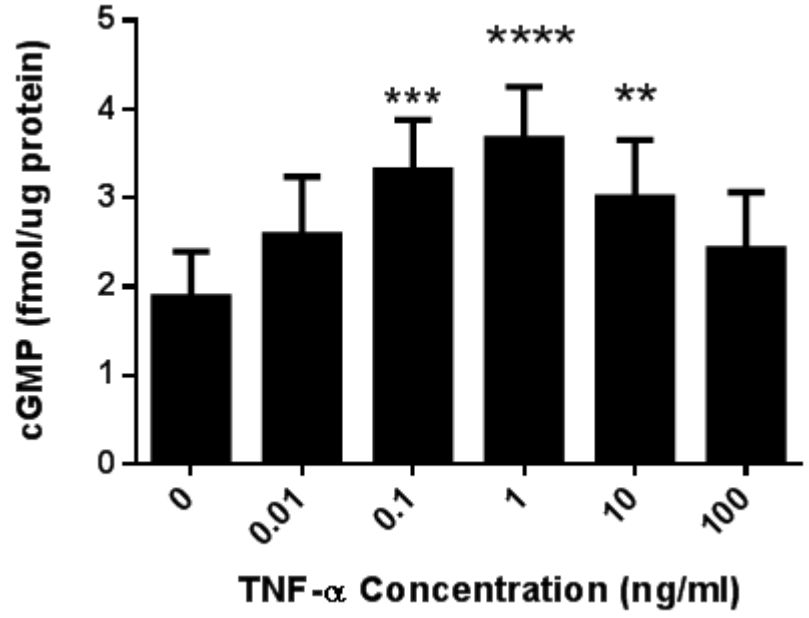

0
10
20
30
40
50
% Relaxation of PHE-induced tone
-9
-8
-7
-6
-5
[CCH] (M)
TNF-α + Insulin
Control

Figure 6

A


cGMP (fmol/ug protein)
0
1
2
3
4
5
0
6
12
18
24
Time (hr)
***
*
*
TNF-α
Control
Insulin
Insulin & TNF-α

B


cGMP (fmol/ug protein)
0
1
2
3
4
5
***
****
**
0
0.01
0.1
1
10
100
TNF-α Concentration (ng/ml)