PC90

The influence of shear stress on physiological angiogenesis and regression

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Chronic vasodilator treatment intensifies levels of shear stress in capillary beds, stimulating a specific form of angiogenesis, termed longitudinal splitting (Egginton, 2001). The use of the α1-adrenoreceptor antagonist, prazosin is well-established for investigation of this process in skeletal muscle (Baum, 2004). Various studies have explored changes in protein expression during shear stress-induced angiogenesis. However, little attention has focussed on the physiological response to cessation of vasodilator treatment and subsequent regression.

Male mice, of the C57BL/10 strain, received prazosin dissolved in tap water (50mg/L) for a period of 4 weeks. In addition to control animals, time points were considered during prazosin treatment (14, 28 days) and the regression phase (3, 7, 14, 28, 42 days; n=4). At sampling, the tibialis anterior muscles were removed, following stunning and cervical dislocation.

Assessment of capillary-to-fibre was used to confirm the microvascular response. Angiogenesis was demonstrated by a 15% increase in capillarity after 4 weeks of vasodilator treatment. Upon cessation of treatment, an equivalent decrease represented vessel regression. Changes in protein expression were explored using immunoblotting, with membranes being protein-stained to ensure equal loading of samples. ANOVA was used to assess statistical significance.

Interestingly, VEGF levels were seen to decline in response to increases in shear stress, with statistical significance seen ($P<0.01$), supporting the theory of hyperperfusion (Baum, 2004). Despite this decrease, angiogenesis occurred, reinforcing the suggested role of nitric oxide as an angiogenic factor (Williams, 2006). As expected, increases in eNOS levels were seen in response to prazosin treatment. A mirroring decline from peak eNOS levels was seen during the regression phase, reaching significance after 6 weeks ($P<0.05$). The expression of the main angiogenic VEGF receptor, Flk-1, increased with shear stress, perhaps compensating for reduced levels of its ligand.

Interestingly, Ang-2 levels increased bimodally reflecting its pleiotropic effects, with a 23% increase seen after 2 weeks of prazosin treatment. Following a brief decrease, a further 74% increase occurred during vessel regression. The effects of this cytokine were clearly dependent on associated levels of VEGF. We conclude that this form of angiogenesis involves both a rapid phase on induction and regression following withdrawal of stimulus.

All procedures follow current UK legislation

Baum O et al (2004). Am J Physiol. 287, H2300-H2308.

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Authors have confirmed where relevant, that experiments on animals and man were conducted in accordance with national and/or local ethical requirements.

PC91

Effects of diabetes and insulin resistance in pregnant rats on *ex vivo* vascular reaction to magnesium

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Graded, movement-coupled hyaluronan secretion into joints and potential signal pathways *in vivo*

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Hyaluronan (HA) governs interstitial hydraulic permeability. In synovial fluid it also contributes to joint lubrication and intra-articular (i.a.) fluid conservation via filter-cake formation. Since i.a. HA injections and exercise reportedly improve moderate osteoarthritis, we investigated the effect of graded joint movement on HA secretion and potential signal pathways *in vivo*.

Endogenous HA was washed out from pairs of cannulated knee joint cavities in anaesthetised rabbits (pentobarbitone 30 mg kg^{-1}, urethane 500 mg kg^{-1} i.v.). The joints were subjected to intermittent, passive cycling at a fixed frequency (0.5Hz) for different durations (0, 1, 3, 9min in every 15min, duration 0-60%) or 20% duration at different frequencies (0, 0.17, 0.5, 1.5Hz) to determine stimulus-response curves. Newly secreted HA was harvested by washout after 5h and analysed by HPLC. Putative signalling pathways were assessed using i.a. pharmacological agents and contralateral control vehicle in moved or static joints.

Movement at 0.5Hz and 20% duration almost doubled the HA secretion rate qHA ($p<0.0001$, paired t test, n=35). The coupling was a graded one, with curvilinear stimulus-response curves to both frequency and duration of movement ($p=0.0001$, ANOVA). Since stretch stimulates HA secretion in cell culture via a Ca^{2+} - protein kinase C (PKC) – MEK-ERK pathway (1), we next probed the pathway's functionality in vivo. The Ca^{2+} ionophore ionomycin more than doubled qHA in static joints ($p=0.02$, paired t test, n=5), as did PKC activation by phorbol ester (PMA) ($p= 0.001$). Moreover the PKC inhibitor bisindolylmaleimide I (BIM) substantially inhibited PMA-stimulated secretion in static joints ($p<0.02$, n=10,16, t test), as did the MEK-ERK inhibitors U0126 and PD98059 ($p\leq0.001$, n=5 respectively, paired t test). Despite these positive results in static joints, BIM, U0126 and PD98059 each failed to inhibit movement-stimulated HA secretion significantly ($p=0.96,0.23, 0.32$ respectively, n=5,8,11, paired t test). By contrast, the phospholipase C (PLC) inhibitor U73122 almost totally abolished the stimulation of

secretion by movement, halving qHA in moved joints (p=<0.001, paired t test, n=10). U73122 did not significant inhibit qHA in static joints (p=0.13, n=5, paired t test).
The study showed for the first time that the coupling between joint usage and HA secretion reported in (2) is a graded one, and that PLC may be an obligatory step in the signal transduction pathway. Ca^{2+} store release, PKC activation and downstream MEK-ERK can stimulate HA secretion in vivo, as in vitro; but whereas the PKC-MEK-ERK pathway mediates the response to stretch in vitro, it did not mediate the response to movement in vivo. Further elucidation of the signalling pathways is needed, with potential therapeutic implications.

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PC93

Commonly used loading controls in Western Blot studies are not suitable for use in post-natal rat skeletal muscles

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Western blot analysis is a widely used method for the semi-quantitative determination of the concentration of specific proteins in a tissue. To control and correct for equal protein loading error, a protein with relatively constant expression in the tissue is normally used as an internal loading control. In most studies the two main proteins commonly used are actin and α-glycerophosphate dehydrogenase (α-GAPDH) (Dittmer and Dittmer, 2006). However, in contrast to other proteins actin is too abundant in skeletal muscle and α-GAPDH is known to vary with fibre type. Therefore, the primary aim of this study was to investigate whether actin, β-tubulin and α-GAPDH are suitable loading controls for differentiating rat skeletal muscles. The experiments were performed using the extensor digitorum longus (edl, a mainly fast-twitch muscle in adult rats) and the soleus (a predominantly slow-twitch muscle in adult rats) muscles of Wistar rats aged between 1 and 90 days. The rats were killed by CO_2 inhalation and the EDL and soleus muscles from both hind limbs were dissected and rapidly snap-frozen in liquid nitrogen. Proteins were then extracted using NP40 cell lysis buffer. Equal amounts of protein (~10 μg per lane) were then resolved in a 10% polyacrylamide/sodium dodecyl sulphate gel. The proteins were then identified using monoclonal antibodies raised against β-tubulin, actin and α-GAPDH and analysed using Scion Image from NIH. The results show that the concentrations of all three proteins increase with age and that this is most rapid between the age of 1 and 14 days. Thereafter, the concentrations of actin and β-tubulin tended to remain relatively constant and were basically similar in the edl and soleus. On the other hand, the concentration of α-GAPDH was always higher in the edl than in the soleus at all of the ages examined. From these results we suggest that actin, β-tubulin and α-GAPDH are not suitable loading controls for skeletal muscles isolated from animals younger than 14 days.

Dittmer A & Dittmer J (2006). β-actin is not a reliable loading control in Western blot analysis. Electrophoresis 27, 2844-2845.

This research was funded by the University of East Anglia

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L-Carnitine supplementation attenuates intermittent hypoxia-induced oxidative stress and delays muscle fatigue in rats

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The concept of L-carnitine supplementation to improve muscular performance is based on the role of L-carnitine in the rate limiting step of β-oxidation of fatty acids (1). L-carnitine attenuates free radical induced oxidative stress during recovery after exercise stress (2) and in pathological conditions (3). Thus it was hypothesized that L-carnitine may reduce intermittent hypoxia induced oxidative stress and thereby delays muscle fatigue. Thirty-six adult male Sprague Dawley rats were divided in two batches and each comprising three groups (n=6/group)-unexposed control; intermittent hypoxia exposed (6 hrs/day for continuous 7 days), intermittent hypoxia exposed (6 hrs/day for continuous 7 days) with L-carnitine supplementation (100mg/kg body weight/ day for 7 days). After the completions of exposure in batch I, rats were anaesthetized with ketamine (50 mg/kg bw, i.p.) and xylazine (10mg/kg bw, i.p.) and sacrificed. The thiobarbituric acid reactive substances, protein carbonyl and lipid hydroperoxides were estimated in the muscle tissue to investigate the efficacy of carnitine in attenuating oxidative stress. In batch II experiment, rats were anaesthetized with intramuscular dose of ketamine (50mg/kg bw). The gastrocnemius muscle of right hind limb with intact sciatic nerve was dissected with the Achilles tendon and connected to a force transducer. The gastrocnemius muscle was set at a length which produced an optimal force during each tetanic contraction. Electrical stimulation was used to induce six tetanic muscular contractions in gastrocnemius muscle after completion of exposure. Percentages of mean performed work, time of decay to 50% of peak force of contraction, and peak force of contraction were measured during tetanic contractions using high-speed data acquisition system. Mean frequency during recovery between tetanic contractions was measured from electromyography. Muscle damage was indirectly measured from plasma creatine kinase and lipid hydroperoxides. Significant reduction in thiobarbituric acid reactive substances, protein carbonyl, lipid hydroperoxides, and creatine kinase activity in L-carnitine supplemented-intermittent hypoxia exposed group when compared with placebo treated-intermittent hypoxia exposed group suggests that L-carnitine reduces oxidative damage and thereby delays muscular fatigue, which was further evident from improvement in maximum force of tetanic contraction, time of decay to 50% of peak force of tetanic contraction