The effect of moderate maternal postconceptional undernutrition and undernutrition in early postnatal life on mesenteric vascular reactivity in adult sheep

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The pre- and post-natal nutrient environments are implicated in the development of cardiovascular disease in later life (Eriksson et al. 2001). In sheep, undernutrition in early gestation alters fetal peripheral vascular reactivity (Ozaki et al. 2000; Nishina et al. 2003) and cardiovascular control in fetal and early postnatal life (Hawkins et al. 2000). However, to date, there is little information in sheep on the effect of diet in early gestation on vascular function in adulthood or how this is affected by postnatal nutrition.

Pregnant Welsh Mountain ewes (Animals (Scientific Procedures) Act 1986) received either 100% nutrient requirements throughout gestation (control C, n=13) or a 50% total reduction in total nutrient intake for 30 days post conception, and 100% nutrient requirements thereafter (Restricted, U, n=12). At weaning, a subset of the offspring from each group were fed ad libitum (CC, n=5 & UC, n= 5) or a restricted diet (CU, n=8 & UU, n=7) to reduce body weight to 85% of individual target weight (predicted from 0 - 12 week growth trajectory) between 12 and 25 weeks, and ad libitum thereafter. At 2.5 years of age sheep were killed humanely with sodium pentobarbitone (160 mg/kg i.v.) and set of the offspring from each group were fed nutrition.

We propose that an increase in oxidative stress in a model of ‘in utero programming and catch-up growth’, leads to abnormally high levels of telomere attrition in kidney (Jennings, 1999) and reduced longevity (Ozanne, 2004). Dysfunctional mitochondria are thought to be a major contributor to the production of reactive oxygen species (ROS). The aim of this study was to measure mitochondrial respiratory chain complex activity (I, II, III, II+III, IV) and reduced glutathione (GSH) in the kidney of 12 month male offspring.

Mitochondrial dysfunction precedes telomere shortening and premature death in a model of developmental programming

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We propose that an increase in oxidative stress in a model of ‘in utero programming and catch-up growth’, leads to abnormally high levels of telomere attrition in kidney (Jennings, 1999) and reduced longevity (Ozanne, 2004). Dysfunctional mitochondria are thought to be a major contributor to the production of reactive oxygen species (ROS). The aim of this study was to measure mitochondrial respiratory chain complex activity (I, II, III, II+III, IV) and reduced glutathione (GSH) in the kidney of 12 month male offspring.

Wistar rats were fed on dietary regimes as described in Ozanne (2004): control group (C), n=12, 20% protein during pregnancy, 20% protein during lactation, and chow from weaning through to adulthood; recuperated group (R), n=14, 8% protein during pregnancy, 20% protein during lactation, and chow from weaning through to adulthood. Male offspring were humanely killed at 12 months, and kidney cortex and medulla separated. Mitochondrial complex I to IV and citrate synthase activities were measured as described in Bolanos (1995). Results were normalized as a ratio of protein content to compensate for mitochondrial respiratory chain complex activity (I, II, III, II+III, IV) and reduced glutathione (GSH) in the kidney of 12 month male offspring.

Mitochondrial complex I to IV and citrate synthase activities were measured as described in Bolanos (1995). Results were normalized as a ratio of protein content to compensate for mitochondrial respiratory chain complex activity (I, II, III, II+III, IV) and reduced glutathione (GSH) in the kidney of 12 month male offspring.

In the cortex a significant down regulation in R of mitochondrial respiratory chain enzyme activity, citrate synthase enzyme activity and GSH was observed using a paired t-test in standardised results; citrate synthase; C (136±6.6) vs. R (119±3.4) p<0.01, complex II+III; C (41±3.1), R (24±3.3) p<0.001, complex II; C (93±3.9) vs. R (83±3.2) p<0.05, complex IV; C (3.2±0.3) vs. R (2.4±0.1) p<0.01, GSH; C (1.1±0.4) vs. R (0.7±0.5) p<0.001. In the medulla a significant reduction was only observed in GSH measurements; C (0.8±0.03) vs. R (0.6±0.03) p<0.001, data means±S.E.M.
We conclude that in utero protein restriction leads to mitochondrial abnormalities, which could conceivably precede and be a contributory factor in telomere shortening and premature death in these animals.


This project was funded by the British Heart Foundation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Reoxygenation-induced calcium overload in coronary endothelial cells: Does the endoplasmatic reticulum play a key role?
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Endothelial cells (EC) cover the entire inner surface of the vessel system. They function as a selective barrier to protect the surrounding tissue from extravasations. Failure of this endothelial barrier caused by ischemia leads to oedema. During ischemia a rise in cytosolic calcium and a formation of gaps can be observed. These processes are further aggravated during the early period of reperfusion (‘reoxygenation injury’). Former studies have shown that removal of extracellular calcium reduces the additional increase, but the calcium cannot be reduced to its original level. Could this be due to an internal release mechanism of calcium? To address this question, we monitored with optical imaging the cytosolic calcium level in living cardiac endothelial cells during reperfusion with a focus on the main intracellular store, the endoplasmatic reticulum.

Microvascular coronary EC from humanely killed rats were exposed to simulated ischemia (40 min, pH 6.4) followed by reoxygenation (40 min, pH 7.4, 2.5 mM glucose). Cai was monitored via fura-2 fluorescence. The SERCA-inhibitor thapsigargin (THG, 10nM), the IP3-release-channel-inhibitor xestospongicin (XeC, 3µM), the phospholipase C inhibitor U73122 (1µM), its inactive analogue U73433 (1µM) or the scavenger trolox (250µM) was applied during reperfusion. The data presented describe the mean values ± s.e.m. in arbitrary units of fluorescence intensity. They were taken from at least four different experiments.

In the absence of these substances the fura 2-ratio significantly increased during reoxygenation from an end-anoxic value of 1.28±0.03 to 1.40±0.03 after 40 min (n=124 cells; p<0.05 vs end-anoxia). Emptying the calcium store by inhibiting its re-entry by THG reduced in the presence of extracellular calcium after 40 min the calcium rise versus control (Fura-2 ratio: 1.26±0.02; n=120 cells; p<0.05 vs end-anoxic). This suggests an involvement of ER during reoxygenation. Inhibition of the IP3-mediated calcium release by XeC abolished the additional Cai rise during reperfusion (1.26±0.03; n=93 cells; p<0.05 vs without XeC). This indicates that the IP3-receptor dependent release is involved in the reperfusion-induced rise in Cai. Application of the phospholipase C inhibitor U73122 also reduced the Cai rise (1.29±0.03; n=112 cells; p<0.05 vs without U73122) whereas its inactive analogue (1.43±0.02; n=123 cells; ns vs without U73433) had no effect. Trolox, a vitamin E analogue, prevented the additional Cai rise (1.30±0.03; n=98 cells; p<0.05 vs without Trolox).

In conclusion, the increase in cytosolic calcium during reoxygenation is due to an influx of extracellular calcium. Additionally, an IP3 dependent calcium release from the ER occurs. The activation of the IP3-release-channel is mediated via activation of phospholipase C. This activation is probably caused by the generation of ROS during reoxygenation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Human umbilical cord blood stem cells differentiate into endothelial cells
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The aim of this study was to explore the capacity of human CD34+ stem cells from umbilical cord blood to differentiate into endothelial cells, in culture, after addition of specific growth factors, and to also compare their behaviour with that of endothelial cells obtained from human umbilical vein (HUVEC).

CD34+ cells were positively selected using immunomagnetic beads (Dynal, Oslo-Norway). Isolated cells were grown in medium containing rh Stem Cell Factor (50 ng/ml), rh GM-CSF (10 ng/ml), rh IL-3 (10 ng/ml), 1% methylcellulose in Iscove MDM, 30% FCS, supplemented with antibiotics and 0.75 mg/ml Endothelial Cells Growth Supplement (ECGS). The cells were seeded in 24-well plates and incubated at 37 °C in an atmosphere with 5% CO2. After 3 days of culture, beside 0.75 mg/ml ECGS, the medium was also supplemented with Epidermal Growth Factor (EGF; 0.1 ng/ml) and beta-Fibroblast Growth Factor (beta-FGF; 1 ng/ml). In another experiment, ECGS was replaced by VEGF (10 ng/ml).

In both experiments, after 10-15 days of culture, the cells formed small clusters of adherent, flat, and elongated cells, finally forming a monolayer that filled the plate. Immunocytochemical analysis revealed the presence of CD31 surface antigen and von Willebrand factor. Flow cytometric analysis of ECAM expression of the CD34+ cells displaying morphological alterations showed that 9.6±2.0% of the studied cells expressed CD34+ (ICAM-1 and 7.7±3.3% expressed CD106/VCAM. Endothelial cells obtained from umbilical vein presented a similar expression of the studied endothelial adhesion molecules before the action of TNF. After 10 h incubation with TNF, the expression of CD54 and CD106 was not significantly increased on cells obtained from CD34+ stem cells (10±0.2 and 7.9±0.1%, respectively), whereas HUVEC expressed CD54 and CD106 in proportion of 43±2 and 29±1.4%, respectively. These data demonstrate that a fraction
of CD34+ cells develop some endothelial cell characteristics when cultured with ECGS or VEGF, but they are functionally different from HUVECs.


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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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VEGF induces but bFGF inhibits nitric oxide production in endothelial cells

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Knockout mice for bFGF (basic fibroblast growth factor) show reduced systemic blood pressure (Dono et al. 1998). The reason for that, however, is not completely understood so far. We hypothesized that nitric oxide (NO) plays a modulator role in angiogenesis and vascular remodeling by interactions with growth factors and investigated therefore the interplay of NO with bFGF and VEGF (vascular endothelial cell growth factor), both cytokines known for their potency in vascular remodelling and angiogenesis and additionally investigated possible modulator effects of both, bFGF and VEGF, on NO synthesis as possible feedback loop.

Primary endothelial cells (HUVEC) were used throughout the study. VEGF (5-10ng/ml) or histamine (1µg/ml), used here as independent control, induced NO production after 6h (measured as NO₂⁻ (nitrite) 3-fold vs. control, n=8, p<0.05) significantly, whereas bFGF (3ng/ml) had no inductor effect. Furthermore, bFGF even inhibited NO production when it was combined with VEGF or histamine. These bFGF effects were abolished by a bFGF receptor antagonist or wortmannin, indicating a PI3Kinase-dependent signalling pathway. Similarly, in shear stress experiments (16dyn/cm²) the addition of bFGF receptor blockers led to an exponential increase in shear stress induced NO formation indicating, again, a bFGF-dependent inhibition of mechanically induced NO formation. The inhibitory effects of bFGF and the inductive effects of VEGF on the NO production were also reflected by their individual interplay with NO. Exogenously added NO (S-nitroso-N-acetyl-D,L-penicillamine, SNAP, 1µM,) inhibited bFGF-induced migration by 50% but not the VEGF-induced (n=4x10, p<0.05). Accordingly, bFGF-induced differentiation (capillary-like structure formation from aortic rings) was increased 2-fold if basal NO production was inhibited (L-NA (L-nitro-arginine), 30µM) whereas the effect of VEGF on vessel sprouting was reduced by 50%. Similar effects were found in proliferation in which VEGF- but not bFGF-induced cell growth was reduced by L-NA (14% after 72h, n=8, p<0.05).

The results identify NO as a significant modulator of vascular remodelling and angiogenesis by affecting the efficiency of the growth factors VEGF and bFGF in an opposed manner. Moreover, bFGF-dependent inhibition of NO production might be a set back mechanism for flow-induced NO production. By that, bFGF prevents an overshoot in dilatation and may participate in local blood pressure regulation.


Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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Anti-angiogenic, inhibitory splice variants of VEGF (VEGFxxxb) are downregulated in pre-eclamptic placentae

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Pre-eclampsia is characterised by hypertension, proteinuria, oedema (including cerebral oedema) and placental under-perfusion, attributed to reduced angiogenesis in the placenta. Vascular Endothelial Growth Factor (VEGF) is upregulated in serum and plasma from pre-eclamptic patients. There are two families of VEGF produced by alternative splicing; an angiogenic family, which causes vaso-dilatation and increased permeability, and an inhibitory family that inhibits blood vessel growth and vasodilatation. The isoform type of VEGF that is upregulated in pre-eclampsia is unknown, so we tested the hypothesis that high VEGF levels in pre-eclampsia are inhibitory VEGF isoforms. Placentae were collected from 21 pregnant women aged 18-37 from Gloucester Royal Hospital and Southmead Hospital, Bristol, UK. The protocol for this study was approved by the local Ethical Committee and informed consent obtained from all patients. 8 subjects were diagnosed with pre-eclamptic toxemia, defined as systolic blood pressure above 140 mmHg and diastolic pressure above 90 mmHg, proteinuria (>1000mg per 24h collection) with resolution of both hypertension and proteinuria by 12 weeks postpartum. Samples from healthy, normotensive pregnant women were taken at term and included as controls. Protein was extracted from the placentae, quantified and subjected to Western blotting and ELISA using an antibody that detects all isoforms of VEGF (pan-VEGF), and one that is specific to the anti-angiogenic family-VEGFxxxb. VEGFxxx levels were calculated as the difference between total VEGF and VEGFxxxb.

Western blots probed with a pan VEGF antibody showed strong staining of bands at approximately 28, 38 and 44 kDa, corresponding to dimers of VEGF121, VEGF165 and VEGF189. When blots were probed with antibodies to VEGFxxxb, the same pattern was seen in all samples, with bands at 38 and 44 kDa showing up strongly, and 28 kDa clear, but less strong. ELISA of the protein showed lower expression of VEGFxxxb in normal (0.97±0.19ng/mg) than pre-eclamptic (1.67±0.32ng/mg, p=0.06) placentae. VEGFxxxb expression however was lower in pre-eclamptic (7.1±0.8pg/ml) than in normal samples (11±1.3pg/ml, p<0.05, Mann Whitney U test). The mean percentage of total
VEGF that was VEGF_{165b} decreased from 1.48±0.24% in normal placentae to 0.66±0.2% in pre-eclamptic patients (p<0.05). In summary VEGF_{165b} is downregulated in pre-eclampsia. It makes up a very small proportion of total VEGF in placenta unlike in other tissues so far investigated (e.g. prostate, colon and vitreous) and is therefore unlikely to contribute to the symptoms of pre-eclampsia. VEGF_{165b} downregulation is probably rather a sequelae of tissue underperfusion in the placenta.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.
A novel method of assessing coronary endothelial function using thermodilution

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Invasive clinical assessment of endothelial-dependent coronary microvascular function currently relies on intracoronary Doppler flow measurements and quantitative coronary angiography (QCA) to derive changes in coronary blood flow in response to endothelial agonists [1]. However, this method can be technically challenging with poor reproducibility. We hypothesised that changes in coronary flow derived by a thermodilution method, using the pressure wire (which can function as an intracoronary dual pressure-temperature sensor), could be used to reliably assess coronary microvascular endothelial function. The transit time (Tmn) of a bolus of room temperature saline using the latter technique is known to be inversely proportional to coronary flow [2].

Twenty patients undergoing PCI to a single vessel were recruited and an adjacent coronary vessel free of significant disease was studied. We compared the percentage change in absolute coronary flow from baseline using Doppler/QCA with the percentage reduction in Tmn using thermodilution in response to a 2 min intracoronary infusion of substance P (20pmol/min). Thermodilution and Doppler derived values were compared using linear regression analysis.

There was a very close correlation (R²=0.75, p=0.0001) between percentage change in absolute blood flow in response to substance P (as measured by a Doppler flow wire) and reduction in Tmn (thermodilution) as measured with the pressure wire. This simple thermodilution technique can be used to reliably assess endothelium-dependent changes in coronary blood flow and hence microvascular endothelial function. The results correlate well with the current gold standard using the Doppler flow wire.


Where applicable, the experiments described here conform with Physiological Society ethical requirements.

Endothelial function and systemic inflammation in healthy subjects with the metabolic syndrome

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The metabolic syndrome (MS) as defined by the National Cholesterol Education Programme Adult Treatment Panel III (ATP-III) predisposes to the formation of atherosclerosis [1]. Multiple previous studies have demonstrated that vascular endothelial dysfunction (ED) and systemic inflammation can act as independent predictors of future cardiovascular (CVS) complications [2,3]. We have investigated the relationship between ED/inflammation in healthy subjects with/out the ATP-III criteria for the MS.

Sixty four (26 male; mean age (years) with MS 40.7±10.9, without MS 34.7±6.3) healthy non-diabetic non-smokers were screened for MS and divided into two groups depending on the presence/absence of MS. Inflammatory markers (C-reactive protein (CRP), tumour necrosis factor-alpha receptor-2 (TNF, a marker of TNF-alpha activity), interleukin-6 (IL-6)) brachial artery flow mediated dilation (FMD, a measure of endothelial function), insulin resistance (using the homeostasis assessment (HOMA)) [4] were compared in both groups. CRP was measured using high-sensitivity turbidimetric immunoassay and TNF/IL-6 using enzyme-linked immunosorbent assay. High resolution ultrasound was used to assess FMD. Brachial artery diameter was measured at baseline, blood flow occluded with a pneumatic cuff in the forearm for 5 min and artery diameter re-measured approximately 40 s after deflation of the cuff. FMD was expressed as the percentage change in brachial artery diameter from baseline.

Values are presented as mean ± S.D. Student’s t test was used to analyse differences between subjects with/without MS. Analysis of variance with linear trend was used to assess correlation between variables and ATP-III diagnostic criteria. Statistical significance was accepted at p<0.05.

FMD values were significantly lower and TNF, IL6 and HOMA higher in subjects with MS. There was no difference in CRP levels. One-way analysis of variance showed a significant linear trend between FMD (p<0.001, R²=0.25) and a positive trend between logHOMA (p<0.001, R²=0.40), logIL6 (p<0.001, R²=0.23) and log TNF (p=0.01, R²=0.10) and the number of diagnostic characteristics of the ATP-III criteria present in each subject.

Healthy subjects with the MS have greater ED and systemic inflammation in comparison to healthy subjects without the MS. These changes have independently been shown to be associated with an elevated risk of future CVS complications. Furthermore, the extent of endothelial dysfunction, insulin resistance and inflammation increases with the presence of each additional ATP-III diagnostic criteria. We did not identify a significant difference in CRP levels between the two groups.

Endothelial function, systemic inflammation and insulin resistance in subjects with and without the ATP-III criteria for the metabolic syndrome (MS)

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS n=18</th>
<th>No MS n=46</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD % change</td>
<td>6.2±2.0</td>
<td>8.8±3.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL6 (pg/ml)</td>
<td>3.2±2.3</td>
<td>1.7±1.8</td>
<td>0.02</td>
</tr>
<tr>
<td>TNFalpha (ng/ml)</td>
<td>210±587</td>
<td>160±391</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>3.5±4.5</td>
<td>4.3±6.7</td>
<td>NS</td>
</tr>
<tr>
<td>HOMA</td>
<td>3.9±1.0</td>
<td>1.6±0.2</td>
<td>0.01</td>
</tr>
</tbody>
</table>

There is a significant difference in FMD, TNF, IL-6 and HOMA values between subjects with/without MS. Values are presented as mean±S.D.

Mibefradil blocks agonist and store depletion induced Ca\(^{2+}\) influx in smooth muscle of rat retinal arterioles

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Multiple Ca\(^{2+}\)-channels mediate Ca\(^{2+}\) store re-filling and agonist induced Ca\(^{2+}\) influx in smooth muscle. We report here that mibefradil, an inhibitor of T type voltage-activated Ca\(^{2+}\)-channels, can also inhibit agonist and store mediated Ca\(^{2+}\)-entry in arteriolar myocytes. Arterioles (20-40 µm outside diameter) were freshly isolated from retinae from humanely killed rats and loaded with fura 2/AM. Mean cytosolic Ca\(^{2+}\) of the smooth muscle cells was estimated by microfluorimetry using 340/380 nm excitation. Single arteriolar fragments were superfused with Ca\(^{2+}\)-free Hank’s solution and the rate of Ca\(^{2+}\) influx estimated from the rise in fluorescence ratio following re-introduction of Ca\(^{2+}\). Three Ca\(^{2+}\) influx pathways were studied: (i) That due to store re-filling after store depletion with cyclopiazonic acid (10 µM, CPA), (ii) agonist induced Ca\(^{2+}\) influx generated by endothelin-1 (Et-1, 10nM) and, (iii) influx initiated by KCl (75 mM). In 1 mM Ca\(^{2+}\), each agent increased Ca\(^{2+}\) influx rate 8-100 fold in different microvessels compared to control. Mibefradil inhibited, dose dependently, all three types of Ca\(^{2+}\) influx pathway at low concentrations with IC\(_{50}\)s of 0.2 nM (CPA), 1.2 nM (Et-1) and 6.0 nM (KCl) and 88-96% block at 30 nM (3-5 microvessels from 3-5 animals for each concentration).

Perfused arteriole clamped recordings were made from single smooth muscle cells within microvessels. No T-type Ca\(^{2+}\) currents were detected on depolarising voltage steps from a holding potential of -100mV even in the presence of a divalent free solution. The retinal arterioles exhibited L-type Ca\(^{2+}\) currents and these were activated by the non-dihydropyridine agonist, PPL-64176 (1 µM). Ca\(^{2+}\) influx rates exceeded the temporal resolution of the drug delivery system (1 s), so Ca\(^{2+}\) influx was studied by introducing 30 mM instead of 1 mM Ca\(^{2+}\). 30 mM mibefradil only reduced this influx rate by 19+2% (n=3). These results suggest that non-voltage operated Ca\(^{2+}\)-channels are at least as sensitive to inhibition by mibefradil as classical T type channels.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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D-Glucose inhibits system y\(^{+}\)L transport activity via activation of protein kinase C in human umbilical vein endothelium

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Nitric oxide (NO) is synthesized from the cationic amino acid L-arginine. Transport of L-arginine occurs mainly via the family of cationic amino acid transporters (CAT), system y\(^{+}\)/CAT, and via system y\(^{+}\)L (Deves & Boyd, 2000). System y\(^{+}\)L exhibit very high affinity, exchanges cationic by neutral amino acids in presence of Na\(^{+}\), and is a heterodimer formed by a heavy chain (4F2hc) and a light chain (4F2-lc2 or 4F2-lc3) of the surface antigen 4F2. Elevated D-glucose modulates system y\(^{+}\)/CAT activity and expression in human umbilical vein endothelium (HUVEC), however there are not reports regarding modulation of system y\(^{+}\)L by D-glucose. We determined whether expression and activity of system y\(^{+}\)L is modulated by D-glucose and its potential role in the regulation of L-arginine/NO pathway in HUVEC.

Cells were cultured in medium 199 containing 20% bovine sera, 3.2 mM L-glutamine, 100 IU/ml penicillin-streptomycin (37°C, 5% CO\(_2\)). Passage 2 cells were exposed (0-24 h) to medium containing 5-25 mM D-glucose. L-Arginine transport (L-[\(^{3}\)H]arginine, 0.1-20 µM, 6.6 Ci/mmol) was measured in absence or presence of L-leucine (1 mM), N-ethylmaleimide (NEM, 200 µM, system y\(^{+}\)/CATs inhibitor), 12-myristate 13-acetate [PMA, 100 nM, protein kinase C (PKC) activator], calphostin C (100 nM, PKC inhibitor) or PD-98059 [10 µM, MAP kinase kinase 1 and 2 (MEK1/2) inhibitor]. PKC activity was monitored by phosphorylation of a PKC substrate. Experiments were done in Krebs solution with or without Na\(^{+}\). SLC3A2/4F2hc, SLC7A7/4F2-lc2 and SLC7A6/4F2-lc3 genes encoding for system y\(^{+}\)L were detected by RT-PCR.

Transport of 1.5 µM L-arginine was inhibited (P<0.05, unpaired Students t-test, n=8-12) by L-lysine, L-leucine and L-phenylalanine, but was unaltered by L-alanine or L-cysteine. NEM did not alter 1.5 µM L-arginine transport, but inhibited (92±3%, mean ±S.E.M.) transport of 100 µM L-arginine. L-Arginine transport in medium containing 1.5 µM L-arginine was inhibited by D-glucose, but inhibited (92±3%, mean ±S.E.M.) transport of 100 µM L-arginine. L-Arginine transport in medium containing 1.5 µM L-arginine was inhibited by D-glucose, but inhibited (92±3%, mean ±S.E.M.) transport of 100 µM L-arginine. L-Arginine transport in medium containing 1.5 µM L-arginine was inhibited by D-glucose, but inhibited (92±3%, mean ±S.E.M.) transport of 100 µM L-arginine. L-Arginine transport in medium containing 1.5 µM L-arginine was inhibited by D-glucose, but inhibited (92±3%, mean ±S.E.M.) transport of 100 µM L-arginine.
Angiogenesis in oxidative and glycolytic muscle of Wistar rats during prolonged ischaemia

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Whether ischaemia induces angiogenesis in skeletal muscle is controversial, which may be due to a differential response in capillary supply to tonically active (e.g. oxidative soleus, SOL) and phasically active (e.g. glycolytic extensor digitorum longus, EDL) muscles. It may also depend on the time course of ischaemia, with most studies examining chronic responses alone. Finally, a differential response may occur where there are regional differences in fibre cross sectional area (FCSA) and/or fibre type composition. Consequently, the response of SOL and EDL muscles to progressive chronic ischaemia was investigated at 2, 4 and 6 weeks, using coordinate-dependent sampling to identify specific regions (Deveci et al. 2001). Contralateral muscles were used as control. Male Wistar rats (n=5 for each group; body mass ~250g) underwent hindlimb ischaemia by unilateral ligation of the left common iliac artery under aseptic conditions and anaesthesia (Deveci et al. 2001). Contralateral muscles were used as control. Male Wistar rats (n=5 for each group; body mass ~250g) underwent hindlimb ischaemia by unilateral ligation of the left common iliac artery under aseptic conditions and anaesthesia (combination of xylocaine (3-10 mg kg⁻¹) and ketamine (90 mg kg⁻¹, i.p.)); animals were treated post operatively with antibiotics (0.1 ml s.c. Engeymycin, Deva). Wounds were examined daily with topical antisepctic application (Betadine), and observations made to detect any signs of pain or discomfort (such as staring coat, loss of appetite). Animals were killed by anaesthetic overdose. Muscles were rapidly frozen and histochemical sections stained for alkaline phosphatase to depict all capillaries (Deveci et al. 2001).

Capillary-to-fibre ratio (C:F) significantly increased only after 6 weeks ischaemia in SOL compared to controlateral control muscles (from 2.46±0.08 to 3.15±0.11, mean±S.E.M., P<0.01, ANOVA). There was no ischaemia-induced angiogenesis in EDL over this time course (1.61±0.12 - 1.68±0.07 for 6 weeks, P>0.05). In EDL, there were also no significant alterations in FCSA or CD, either as a whole muscle average or within individual sample regions. However, there was significant fibre atrophy in SOL muscle at 4 weeks (7114±274 - 5905±408 μm², P<0.05), and also significantly increased CD at both 4 and 6 weeks (362±15 - 458±22 mm², P<0.01). It is well known that oxidative activity of SOL muscle is higher and FCSA (average 692±347 μm) larger than glycolytic EDL (average 432±327 μm²). Therefore, these data show that the muscle with the higher oxidative capacity, larger FCSA and is also tonically active (SOL) was more affected by ischaemia leading to angiogenesis, compared with the muscle which has low oxidative capacity, smaller FCSA and is phasically active (EDL). In conclusion, endogenous angiogenesis in skeletal muscle depends not only on the time course of ischaemia, but also multifactorial aspects of muscle phenotype.


The author is grateful to Dr Stuart Egginton for his generous help during this study and to the Physiological Society for a travel grant.

Effect of dietary isoflavones on blood pressure and vascular function in the rat

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Isoflavones, which are found in high concentrations in soy protein, are proposed to be cardioprotective. It has been demonstrated in female spontaneously hypertensive rats, that soy extracts lowered blood pressure, and ovariectomized rats fed a diet rich in isoflavones have improved endothelial function. The aim of this study was to investigate the cardiovascular effects of soy-derived isoflavones in male and female rats, using diets identical in nutrient composition except with regard to the isoflavone content.

Male and female Wistar rats were fed either a low (LI) (0.04 mg/g) or high (HI) (0.2 mg/g) isoflavone-containing diet and mated. Offspring were weaned onto and maintained on the same diet as their dam and sire. At 6 months of age, animals were implanted (under 4% isofluorane in oxygen anaesthesia) with a radio-telemetric cardiovascular monitoring device (DSI, USA). Data were collected every 5 min over a 1 week period and 12h day and night averages calculated. Animals were then humanely killed. Third order mesenteric arteries were dissected and mounted on a small vessel myograph (DMT, Denmark). Dose-response curves to noradrenaline (1x10⁻⁷-1x10⁻³), endothelin-1 (1x10⁻¹⁰-3x10⁻⁸), acetylcholine (1x10⁻⁶-1x10⁻⁵) and nitric oxide (1x10⁻⁷-1x10⁻⁴) were obtained (noradrenaline was used as the pre-constrictor). Vascular function data were assessed using repeated measure ANOVA and one-way ANOVA; radio-telemetric data were assessed using multiple regression with robust standard errors. P<0.05 was considered significant. When results for males and females did not differ, data were combined. Data are presented as the mean ± SEM.

A significant effect of diet on the response to acetylcholine-induced relaxation was observed. Male and female animals fed the LI diet demonstrated decreased sensitivity to acetylcholine compared with animals fed the HI diet (pEC50 LI 6.77 ± 0.1, n=16 versus HI, 7.31 ± 0.1, n=20, P<0.05). There was no difference in systolic (LI 121 ± 1 mmHg n=16, HI 118 ± 1, n=20) or diastolic blood pressure (LI 92 ± 0.7 mmHg n=16, HI 90 ± 1, n=20), heart rate (LI 383 ± 4 beats/min n=16, HI 383 ± 4, n=20) or activity (LI 3.68 ± 0.12 arbitrary units n=16, HI 3.67 ± 0.198, n=20) between the two dietary groups. Feeding a HI diet compared with a LI diet did not alter responses in mesenteric arteres to noradrenaline, endothelin-1 or nitric oxide.

In conclusion, life long consumption of a diet high in isoflavones appears to increase endothelial-dependent dilatation but has no effect on blood pressure or heart rate in 6 month old rats.

We thank special dietary services (Witham, UK) for support and diet manufacture and Solae LLC (St Louis, USA) for providing the soy protein isolate.
Endothelium-dependent and -independent relaxations in aortic rings obtained from hypertensive hooded (Aguti) rats

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Experimental hypertension studies are few in the hooded (Aguti) rat. The present study was designed to investigate the possibility that salt-induced hypertension and/or hypertension due to nitric oxide synthase (NOS) inhibition may be associated with attenuation of relaxation to certain vasodilators.

Hypertension was induced in hooded rats (n=8 each) by administering 8% salt in the diet (Sofola et al. 2003) and/or 100 mg/kg/day Nω-nitro-L-arginine methyl-ester (L-NAME) in the drinking water (Pollock et al. 1993). Aortic rings from the rats were obtained under anaesthesia using a 25% urethane and 1% chloralose mixture given intraperitoneally at a dose of 5mg/kg. They were mounted in a tissue bath containing a physiological salt solution (composition in mM: NaCl, 119; KCl, 4.7; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 15.0; CaCl2, 1.6; glucose, 11.5; pH: 7.4) gassed with a 95% O2-5% CO2 gas mixture and maintained at 37°C. Relaxation responses to acetylcholine, histamine and sodium nitroprusside (SNP) were obtained following precontraction with 10-7M noradrenaline. Relaxation response to SNP was obtained in endothelium-denuded rings. Results are presented as mean ± S.E.M. Statistical analysis was done using one way ANOVA and a post hoc Student-Newman-Keuls test. P<0.05 was taken as statistically significant.

The IC50 of acetylcholine in aortic rings from L-NAME rats (7.9 x 10-7 ± 6.0 x 10-7) was significantly higher than the IC50 in rings from control (9.4 x 10-8 ± 2.8 x 10-8), salt (7.8 x 10-7 ± 4.7 x 10-7) and salt + L-NAME (3.3 x 10-7 ± 2.1 x 10-7) rats (P<0.05). The IC50 of histamine and SNP in the rings from the test groups of rats showed no significant difference from control.

These results suggest that in the hooded (Aguti) rat, endothelium dependent and independent relaxations are preserved in the various forms of hypertension studied except in chronic NOS inhibition where only endothelium dependent relaxation to acetylcholine was attenuated.


Many thanks to the Usman DanFodio University for a study fellowship to F.B.O.M.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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PC114

Serotonin 5-HT2 receptor antagonist improves vasomotor responses in diabetics with atherosclerosis

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Naftidrofuryl improves the blood supply and ischemic damage of the vessel wall by blocking specifically 5-hydroxytryptamine-2 (5-HT2) receptors without influencing the general circulatory bed and improves glucose aerobic metabolism by an action on succinodehydrogenase. The aim was to evaluate the effect of naftidrofuryl on vasomotor dysfunction in diabetic patients with peripheral arterial occlusive disease (PAOD) and coronary artery disease (CAD).

The subjects were 10 type-2 diabetic patients with both PAOD and CAD who received a 600 mg daily dosage of naftidrofuryl orally for 12 weeks. 20 healthy subjects were selected as controls. The patient groups were matched for age, sex and body mass index. The diagnosis of CAD was substantiated by coronary angiography. All the patients had a decreased ankle-brachial index in both legs (0.73 ±0.17). We recorded changes in laser Doppler flux (LDF; PeriFlux 4001, Perimed) induced by a 3 min arterial occlusion on the pulp (apical skin) of the big toe. Basal LDF (b-LDF), postocclusive hyperaemia (m1-LDF), vasoconstrictor response (v-LDF) to deep inspiration (in apical skin), and heat (44°C; PeriTemp 4005) induced hyperaemia (m2-LDF) on the dorsum (non-apical skin) of the big toe were estimated using a PeriSoft programme.

After the therapy the following indices were improved (mean±SD): b-LDF at both locations (apical skin: 48±34 vs 78±42 PU, p<0.005; non-apical skin: 14±9 vs 27±24 PU, p<0.05), v-LDF (13.5±12.4 vs 26.7±15.5%, p<0.0001), m1-LDF (100±54 vs 133±60 PU, p<0.01) and m2-LDF (61±31 vs 95±49 PU, p<0.01).

Our results suggest that 12 weeks of naftidrofuryl therapy improves the cutaneous vasomotor response in diabetic patients with CAD and PAOD.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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PC115

Excess methionine is not responsible for the endothelial dysfunction in offspring of dams fed protein-restricted diet

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In the rat, the restriction of dietary protein during pregnancy leads to maternal vascular dysfunction (Brawley et al. 2004) and
raised blood pressure and endothelial dysfunction in the offspring (Brawley et al. 2003). As the diet is casein based and low in sulphur-containing amino acids, it is usually supplemented with 5 g/kg of methionine (Met). Excessive levels of Met are toxic and can promote homocysteine formation (Clarke & Stansbie, 2001). Thus the relatively high Met to protein ratio in the protein-restricted diet may be important in the maternal effects seen in this model and the subsequent development of endothelial dysfunction in the offspring. The aim of this study was to assess the role of Met in this model.

Pregnant Wistar rats were fed either a control (C; 18% casein, n=10), a protein-restricted diet (PR; 9% casein, n=8) or a protein-restricted diet with only 2.5 g/kg of Met (PR-low Met; 9% casein, n=10) throughout gestation from conception. At 120 days of age, small mesenteric arteries (~250 μm) from humanely killed male offspring were dissected and mounted in the wire myograph in physiological salt solution, at 37°C and continually gassed with 95% O₂ and 5% CO₂. Following normalisation, cumulative concentration-response curves were constructed to phenylephrine (PE; 10 nM - 100 μM); angiotensin II (Ang II; 10 pM - 100 nM), the endothelium-dependent vasodilator acetylcholine (ACh; 1 nM - 100 nM), and the β-adrenoceptor agonist isoprenaline (1 nM - 10 μM). Data are presented as mean ± S.E.M. and differences calculated by one-way ANOVA with Bonferroni post-hoc correction for multiple comparisons. Significance was accepted for p<0.05.

Vasoconstriction to PE and Ang II did not differ between the three groups (p=ns), with the response to Ang II being significantly less than that to PE. Endothelial function as assessed by ACh was not different between the PR and PR-low Met groups (p=ns) both of which were significantly blunted compared to controls (p<0.05). Isoprenaline-induced vasodilatation was not different between the three groups (p=ns).

This study suggests that maternal protein restriction and not high dietary Met is important for the development of endothelial dysfunction in the offspring.

Brawley et al. (2003), Pediatric Research 54, 83-90.
Brawley et al. (2004), J Physiol 554, 497-504.

This work was supported by the British Heart Foundation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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**PC116**

**Gap junctional communication plays a pivotal role for the calcium response of cultured endothelial cells to histamine**

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Previous experiments from our lab have shown that the endothelium-mediated dilatation of resistance arteries to endothelial stimulators was significantly reduced after inhibition of vascular gap junctional communication. We therefore hypothesised that endothelial gap junctions might play a significant role in the net increase of calcium following endothelial stimulation, which ultimately controls the production of endothelial autacoids such as nitric oxide and prostacyclin. Experiments were performed in human umbilical vein endothelial cells (HUVEC) stimulated with histamine (HIS, 5 μM). Studies were performed in n monolayers of HUVEC (2nd passage); values are presented as means ± SEM. Statistical comparisons were performed using t tests. All comparisons reported here were significant (p<0.05). HUVECs were loaded with the calcium sensitive dye Fura 2-AM and the calcium rise in response to histamine was analysed with a camera-based system at intervals of 200 ms. HIS induced an initial Ca²⁺ rise in only 5±0.2% of all cells (n=13 cultures), which were randomly dispersed in the monolayers. A Ca²⁺ rise was finally observed in 98±1% of all cells (n=10) albeit with a delay of up to 3 s. Inhibition of GJ coupling (50μM Meclofenamic acid + 1mM heptanol) did not affect the Ca²⁺ rise in the initially responding cells (in 83±1% identical with controls) whereas the total number of cells responding to histamine was reduced to 38±5% (n=6). Similar results (reduction of the slope of the calcium rise in the monolayer from 131 ± 65±18, n=6) were obtained when the cells were exposed for 3 min to the supernatant of polymorphonuclear neutrophils being stimulated with fMLP (fMLP had no effect on HUVEC GJ coupling, data not shown). In pilot immunohistochemical experiments (n=2) we found that only very few cells in a monolayer were positive for the H1 receptor known to be responsible for HIS-mediated effects in HUVEC.

The Ca²⁺ rise induced by histamine seems to be partly mediated by direct receptor-mediated events occurring in a limited number of ‘pacemaker cells’. In the majority of cells the calcium rise seems to be due to spread of a signal (probably calcium or IP3) via gap junctions to the neighbouring receptor-deficient cells. Thus, control of GJ coupling may be a novel pathway to regulate the EC responsiveness to autacoid-releasing receptor-mediated stimuli.

*Where applicable, the experiments described here conform with Physiological Society ethical requirements.*

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**PC117**

**Two effects of cGMP on vasomotion**

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Cyclic guanosine monophosphate (cGMP) has been shown to be important for the generation of vasomotion in rat mesenteric small arteries. The evidence for the importance of cGMP comes from the observations that removal of the endothelium prevents vasomotion (Gustafsson et al. 1993), prevents synchronisation of the Ca²⁺ activity in the vascular smooth muscle cells (SMCs) (Peng et al. 2001), prevents the depolarisation induced by Ca²⁺ release (Peng et al. 2001), and that all three functions can be restored by adding of 100-300 μM 8Br-cGMP to the bath solution.

In the present study, we have tested whether intermediate concentrations of cGMP partly synchronise SMCs and whether cGMP modifies Ca²⁺ release from SR and hence vasomotion frequency.
Wistar rats (n=33) were humanely killed and the mesentery was removed. Segments of small arteries were mounted for isometric (force) or isobaric (diameter) measurements after removal of the endothelium; spatially and temporally resolved Ca^{2+} was measured with confocal microscopy in the same preparations after loading with Calcium Green-1 AM. After removal of endothelium, no oscillations in tension were seen as reported previously (Peng et al. 2001). At intermediate (10-30 µM) 8Br-cGMP concentrations, a) beating of the oscillatory tension was seen, caused by oscillations with different frequencies in different parts of the artery (n=5), and b) Ca^{2+} imaging revealed islands of cells where Ca^{2+} transients were synchronised within an island but not between islands (7 out of 11 preparations). With 300 µM 8Br-cGMP, coordinated [Ca^{2+}]_i activity and vasomotion were seen. Thus with increasing cGMP concentration, progressively increasing areas of synchronicity were seen, until at 300 µM 8Br-cGMP nearly the entire segment was synchronised. 8Br-cGMP concentration-dependently decreased vasomotion frequency (n=9) and the frequency of unsynchronised [Ca^{2+}]_i waves (n=8) in hyperpolarised SMCs. Thus cGMP progressively reduced the propensity of the intracellular stores to release calcium. We conclude that cGMP has two effects on vasomotion: (a) to enhance intercellular coupling within the vascular wall, promoting vasomotion, and (b) to reduce the frequency of intracellular calcium release, inhibiting vasomotion. These two antagonistic effects may explain the different effects of cGMP in different vascular beds.


Where applicable, the experiments described here conform with Physiological Society ethical requirements.

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**PCI118**

**Inhibition of vascular smooth muscle contraction by the coumarin from Sweet grass**

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Extract from aerial parts of Sweet grass (Hierochloe odorata) are expected to become a source of natural antioxidants for food industry — it contains the new identified coumarin derivative 5,8-dihydroxybenzopyranone, possessing radical scavenging activity (1). No data on the effects of this compound on living organs are available. The effect of the extract fraction, containing 5,8-dihydroxybenzopyranone on smooth muscles tone of blood vessels was investigated.

Adult male guinea pigs, weighting 400-550 g were humanely killed under halothane anaesthesia by cervical transection and exsanguination. Contractility of isolated small mesenteric blood vessels (300-500 µm in diameter) was investigated by small blood vessel wire myography (2). Three different concentrations (1.0 mg/ml, 0.1 mg/ml and 0.01 mg/ml) of Sweet grass extract fraction were added to the organ bath for 30 min of incubation. Contraction of blood vessels was induced by addition of K+-rich solution (80 mM) or α1-receptor agonist phenylephrine (30 µM) before and after the incubation. The endothelium-dependent relaxation was investigated by acetylcholine (1 nM—10 µM) after the precontraction with phenylephrine (30 µM). Results were expressed as mean±SEM and evaluated using t test for paired data.

Vascular contractility was suppressed by the application of extract fraction in a dose-dependent manner (Fig.1). Incubation in 0.1 mg/ml of the extract significantly decreased the force of contraction induced by K+-rich solution and phenylephrine until 37.42±13.23 % (p<0.001; n=8) and until 11.82±6.11 % (p<0.001; n=5), respectively. With an extract concentration of 0.01 mg/ml the contraction force induced by phenylephrine was decreased significantly (p<0.01; n=5) until 41.16±13.47 % then contraction to K+-rich solution was decreased insignificantly (p>0.05; n=8). No changes in endothelium-dependent relaxation were revealed after incubation at 0.01 mg/ml (n=5, p>0.05). Inhibition of NOS by L-NAME (100 µM) had no influence on the decrease of contractility (p>0.05; n=5).

Sweet grass (Hierochloe odorata) extract fraction, containing coumarol derivative 5,8-dihydroxybenzopyranone, inhibits vascular contractility by the direct action on smooth muscles but not on the endothelium.

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**Fig. 1.** Relative change in the contraction force of small blood vessels after the incubation in the different concentrations of extract from aerial parts of sweet grass. Contraction was induced by K+-rich (80 mM) solution (KCl) or 30 µM of phenylephrine (Phe). The contraction force before the exposition of blood vessels in the extract is assumed to 100 %, **p<0.01; ***p<0.001.


Where applicable, the experiments described here conform with Physiological Society ethical requirements.
Does lowered dietary methionine in the low-protein model during pregnancy have a beneficial effect on the rat offspring?

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It has been previously demonstrated that dietary glycine supplementation reverses vascular dysfunction in protein-restricted pregnant rat dams (Brawley et al., 2004) and lowers blood pressure in their offspring (Jackson et al., 2002). Due to the low sulphur content of casein, these diets were supplemented with methionine. The aim of this study was to investigate the effects of reducing dietary methionine in the low-protein diet during pregnancy on both the pregnant dams and their offspring. Three groups of female Wistar rats (n=6) were fed diets of either 18% protein with 0.5% methionine (control: C), 9% protein with 0.5% methionine (protein-restricted: PR) or 9% protein with 0.25% methionine (protein-restricted, low methionine: PR-lowMet) from conception. Food intake and weight gain were measured in the dams. Systolic blood pressure (SBP) and heart rate (HR) were measured using tail cuff in male (n=11-12) and female (n=10-11) offspring at day 28 and 112. Data are presented as mean ± SEM and differences calculated by one-way ANOVA with Bonferroni post-hoc correction for multiple analyses. Significance was accepted for p<0.05.

During pregnancy, average daily food intake of the mother was significantly greater for PR-lowMet than PR dams (p<0.05) and C dams (p<0.01). The PR-lowMet dams also had a greater number of offspring per litter than PR dams (n= 13.0 ± 0.37 versus n= 10.3 ± 0.81) and gained more weight during pregnancy (p<0.05). There was no significant effect on the sex ratio of the offspring.

In this study, protein-restriction did not have a significant effect on either SBP or HR in the male or female offspring, despite evidence of vascular endothelial dysfunction (Torrens et al, this meeting). Reduction of dietary methionine in the low-protein diet during pregnancy lowered SBP in the female offspring at 28 days (p<0.05) (C= 110.9 ± 9.7 mmHg; PR= 120.3 ± 4.7 mmHg; PR-lowMet= 92.4 ± 6.8 mmHg) and there was no effect at 112 days or in the males at either age.

There is a close relationship between the metabolism of glycine and methionine and a methionine load can result in competition for glycine (Meakins et al., 1998). It appears that a diet which is lower in methionine than has previously been used for low-protein studies is more palatable and this may affect pregnancy outcome. However, lowering methionine produced a transient reduction in SBP only in female pups post-weaning. We do not find evidence of longer-term detrimental effects on offspring.

Jackson et al. (2002) Clinical Science 103, 633-639

This work was supported by the British Heart Foundation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

Transgenic ACE mice have reduced ovarian follicle numbers
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The renin-angiotensin system (RAS) has been identified in the ovary of several species. It may be involved in ovarian development, folliculogenesis, steroidogenesis and apoptosis, and may have autocrine/paracrine effects. We studied ovarian follicle numbers in mice with genetic changes to the angiotensin converting enzyme (ACE) gene compared to their wild types (WT).

Ovaries were collected from two strains of transgenic (TG; ACE.3 and ACE.8) mice, produced by homologous recombination in ES cells. These express ACE only in the liver and heart, respectively. Controls were WT animals from the 2 TG strains used in this preliminary study. All animals were humanely killed. The TG mice were aged 9 (ACE.3) and 8 (ACE.8) weeks (n=4 for both TG; WT, n=2 for ACE.3 and n=4 for ACE.8). Ovaries were removed, fixed and serially sectioned. Every tenth section was stained using haematoxylin and eosin and the number of each type of follicle from primordial to preovulatory was then counted. Differences between follicle numbers were analysed by ANOVA.

There was a statistically significant reduction in the number of follicles, from primordial to early antral for ACE.8 TG and to antral for ACE.3 TG mice (see Table 1).

The reduction in follicle numbers seen with both strains of TG ACE mice supports the hypothesis that the RAS is involved in follicle development, particularly the early stages. The reduction in primordial follicles is important as this will influence the number of follicles able to progress to the next stage of development. TG ACE mice will have impaired angiotensin II production, which through its receptors is known to have effects on cell growth, proliferation and regulation. This could explain why the genetically altered mice have fewer follicles progressing to the antral stage.

Impairment of the RAS in the ovary could be a factor for infertility/early menopause, due to the reduction in follicle numbers. Table 1. Ovarian follicle numbers in TG and WT mice

<table>
<thead>
<tr>
<th>Follicle type</th>
<th>TG</th>
<th>SD</th>
<th>WT</th>
<th>SD</th>
<th>P</th>
<th>TG</th>
<th>SD</th>
<th>WT</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>24.6 ± 0.2</td>
<td>29.8 ± 0.4</td>
<td>&lt;0.01</td>
<td>21.3 ± 0.7</td>
<td>31.4 ± 0.5</td>
<td>&lt;0.001</td>
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<tr>
<td>Primary</td>
<td>25.4 ± 0.5</td>
<td>56.3 ± 1.1</td>
<td>&lt;0.001</td>
<td>16.5 ± 1.1</td>
<td>21.8 ± 0.0</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Secondary</td>
<td>42.6 ± 1.9</td>
<td>70.8 ± 0.4</td>
<td>&lt;0.001</td>
<td>40.9 ± 0.2</td>
<td>63.4 ± 0.5</td>
<td>&lt;0.001</td>
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<td></td>
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<tr>
<td>Earliest antral</td>
<td>25.3 ± 0.7</td>
<td>33.3 ± 0.4</td>
<td>&lt;0.001</td>
<td>15.8 ± 0.4</td>
<td>29.3 ± 0.0</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Antral</td>
<td>9.1 ± 0.9</td>
<td>20.0 ± 2.8</td>
<td>&lt;0.01</td>
<td>3.5 ± 0.4</td>
<td>7.9 ± 0.5</td>
<td>&lt;0.05</td>
<td></td>
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<tr>
<td>Preovulatory</td>
<td>0.3 ± 0.4</td>
<td>0.3 ± 0.4</td>
<td>&lt;0.05</td>
<td>0.8 ± 0.0</td>
<td>0.9 ± 0.2</td>
<td>&lt;0.05</td>
<td></td>
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<tr>
<td>Total</td>
<td>127.1 ± 4.1</td>
<td>210.3 ± 3.2</td>
<td>&lt;0.001</td>
<td>97.9 ± 1.2</td>
<td>154.3 ± 1.1</td>
<td>&lt;0.001</td>
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</table>

Where applicable, the experiments described here conform with Physiological Society ethical requirements.