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Effects of vitamin D insufficiency in the fetus and in early life on vascular reactivity in young adult rats

H.C. Parkington[1], S.J. Emmett[1], R. Morley[2], C. Skordilis[1], H.A. Coleman[1] and M. Tare[1], [1]Department of Physiology, Monash University, Victoria 3800 and [2]University of Melbourne Department of Paediatrics, Royal Children's Hospital, Victoria 3052, Australia.

Inappropriate nutrition in fetal and postnatal life can lead to increased cardiovascular risk in the offspring. Vitamin D (vit D) is an important factor little studied in the context of offspring health. The prevalence of vit D insufficiency is increasing in western societies including Australia, and of particular concern, low levels are seen in pregnant women (Grover & Morley, 2001). It has been suggested that maternal vit D insufficiency may increase the risk of autoimmune disease in the offspring, with increased incidence of type 1 diabetes, chronic inflammatory disorders, some cancers, heart disease, high blood pressure, insulin resistance, and elements of Syndrome X. Here we investigated whether vit D deficiency in fetal and early life results in vasodilator dysfunction, as observed in young adult rats.

Female Sprague Dawley rats were fed chow that was vit D deficient (free of added vit D) from 4 weeks of age, with controls fed normal chow (2000U/kg cholecalciferol, vit D). This feeding regime was continued until the end of the study. After 6 weeks, all rats were mated, the litter size reduced to 10 pups on day 4 post-natal, and the pups weaned at 3 weeks of age. Pups from vit D deficient and control dams were maintained on vit D deficient and normal chow, respectively. At 7 weeks of age, a catheter was inserted into the ventral tail artery under isoflurane anaesthesia. Following 2-3 h recovery, arterial pressure was recorded for 1 h. Blood was then obtained via the catheter for serum vit D and Ca^{2+} determinations. The rats were killed by decapitation. The stage of the estrus cycle was determined from a vaginal smear, uterine weight and ovarian inspection. A segment of mesenteric artery, immediately before it entered the wall of the intestine, was mounted on a pressure myograph fitted with an in-line pressure transducer, and pressure set at 57 mmHg without flow. The segment was continuously superfused externally with bicarbonate-buffered physiological saline solution (PSS) at 35°C and 14 ml/min. Segment diameter was recorded using DIAMTRAK® (Neild, 1989). Maximal constriction was determined using 100 mM K^+ PSS and also in response to 10 μM phenylephrine. Endothelium-dependent vasodilation was tested using discrete 2 min applications of acetylcholine in the presence of 70% of maximal tone evoked with arginine vasopressin. Nitric oxide (NO) and prostanoid production was blocked as required using N^{ω}-nitro-L-arginine methyl ester (100 μM) and indomethacin (1μM), respectively.

Serum vit D levels were 8±1 ng/ml in rats fed vit D deficient chow compared with 126±10 ng/ml in controls, and serum Ca^{2+} was halved in deficient animals. Vit D deficient animals were some 20% lighter in weight than normal fed controls. Organ weights were appropriately smaller, except for the brain, in which weight was preserved in vit D deficiency. In vit D deficient rats, conscious blood pressure and heart rate were significantly greater compared with controls (by: 9±3 mmHg and 40±13 beats/min, n=11 in males; 16±4 mmHg and 24±9 beats/min, n=11 in females). Resting tone was doubled in vit D deficiency while the ability to maximally constrict was similar in all groups. Endothelium derived NO vasodilation was halved in vit D deficient males and diestrous females, while the dilation attributable to endothelium-derived hyperpolarising factor (EDHF) was preserved. Conversely, in segments from vit D deprived females in estrus, the dilation evoked by NO was preserved, while that attributed to EDHF was abolished.

These results demonstrate that vit D deprivation in fetal and early life leads to growth retardation and higher arterial pressure in young adult rats. The higher pressure was reflected in elevated resting tone in small mesenteric arteries and marked reductions in endothelium dependent vasodilation, with the vasodilators involved differing depending on the sex steroid status.

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Possible role of the brain angiotensin system in programming and maintaining hypertension in sheep prenatally exposed to dexamethasone

A. McAlinden[1], A. Jefferies[1], M. Cock[1], E. Wintour[1], C. May[2] and M. Dodic[1], [1]Department of Physiology, Monash University, Clayton, Victoria 3800 and [2]Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria 3010, Australia. (Introduced by R. Evans)

Recent studies have generated the hypothesis that a suboptimal intrauterine environment, during a critical stage of development, 'programs' the development of fetal tissues, enabling fetal survival, but with adverse consequences in adult life (Dodic *et al.*, 2002a).

We have shown that elevated mean arterial pressure (MAP) in both male and female adult sheep can be 'programmed' by brief, prenatal exposure to an excess of the synthetic glucocorticoid (GC), dexamethasone (DEX), for only 48 hours, at day 26-28 of the 150 day gestational period (Dodic *et al.*, 1998). Late in gestation (130 days), no elevation in MAP was observed in DEX exposed fetuses, however real-time PCR studies revealed an increase in gene expression levels of angiotensinogen in the hypothalamus and angiotensin II (ANG II) type 1 (AT_1) receptors in the medulla oblongata (Dodic *et al.*, 2002b). When killed at 7 years of age, the sheep prenatally exposed to DEX were found to have increased expression of AT_1 receptors in the medulla oblongata (Dodic *et al.*, 2002b).

Our aim was two-fold: i) to determine if the brain angiotensin system (AS) contributes to the maintenance of elevated MAP; and ii) whether the sensitivity of the brain AS is altered in DEX exposed sheep. Studies were carried out on a cohort of adult male sheep prenatally exposed to either DEX (0.48mg/h) or saline (control) at 26-28 days of gestation. Sheep were instrumented with brain guide tubes (lateral ventricle) and allowed 2 weeks recovery. General anesthesia was induced with an intravenous injection of 5% Sodium Pentothal (0.4mg/kg), then the sheep was intubated and anesthesia maintained with Halothane in 100% oxygen. Cardiovascular function (MAP, cardiac output and heart rate) was measured for one hour (control period), followed by either a four hour intracerebroventricular (icv) infusion of the AT_1 receptor blocker losartan (1mg/h) or artificial cerebrospinal fluid (vehicle). In addition, brain AS sensitivity was tested by measuring cardiovascular function during icv infusions of ANG II (1 or 10μg/h), each dose running for one hour.

Our results show that the MAP response to losartan was similar between the two groups of animals. The MAP response to icv ANG II (1μg/h) was greater ($p<0.05$) in DEX exposed animals compared with the control group. The maximal MAP response to icv ANG II (1μg/h) was higher (ΔMAP=10±1.9mmHg, n=7) in the DEX group compared with the saline group (ΔMAP=6±2.1mmHg, n=7, $P<0.05$). There was no significant difference in MAP response to icv ANG II (10μg/h) between the two groups, however there was a trend towards higher maximal MAP response to ANG II (10μg/h) in the DEX group (ΔMAP=19±1.8mmHg, n=7) compared with the saline group (ΔMAP=14±2.1mmHg, n=7).

These results suggest that the basal brain AS activity does not contribute to the maintenance of elevated MAP in DEX exposed sheep. However, there might be a greater sensitivity of the brain AS to icv ANG II in the DEX exposed animals.

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The effects of maternal renal dysfunction and a high salt diet on the renin angiotensin systems of the pregnant ewe and her fetus

K.J. Gibson, A.C. Boyce, B.M. Karime, C.L. Thomson, J. Wu, Y.P. Zhou and E.R. Lumbers, Department of Physiology and Pharmacology, School of Medical Sciences, University of New South Wales, NSW 2052, Australia.

We previously reported that maternal renal dysfunction, caused by subtotal nephrectomy (STNx) prior to mating, resulted in fetuses with high urine flow rates, high urinary sodium excretions and low haematocrits (Gibson *et al*., 2002). These changes were suggestive of exposure to an increased fluid and solute load from the mother. To determine whether the fetal renin angiotensin system was suppressed, we measured plasma renin levels in 17 STNx and 14 control ewes and their fetuses at 122-128 days gestation (term = 150 days). In addition, we examined the effects of a high salt diet.

At least two months prior to mating, STNx was carried out under general anaesthesia (1 g sodium thiopentone i.v. followed by 1-3% halothane in oxygen). The right kidney was removed and a branch of the left renal artery (supplying at least one third of the kidney) was ligated. At 112-122 days the fetuses and ewes were chronically catheterised under general anaesthesia. No measurements were taken until at least 5 days after surgery. Plasma renin levels were measured as the rate of generation of angiotensin I (ng ml^{-1} h^{-1}) in samples incubated with nephrectomised sheep plasma (a source of angiotensinogen).

Maternal renin levels were similar in the two groups (Control 1.4 ± 0.4 (SE), n=14; STNx 1.2 ± 0.3 ng ml^{-1} h^{-1}, n=17). However, fetal plasma renin levels were lower in the STNx group (6.8 ± 3.0, n=17) than in the control group (15.1 ± 7.9 ng ml^{-1} h^{-1}, n=14, P=0.07).

Six ewes in each group received a high salt diet for 4 days i.e. they had access to 8 l day^{-1} of 0.17 mol l^{-1} NaCl instead of their normal drinking water. When both groups were combined, maternal plasma renin levels fell from 1.9 ± 0.4 to 0.5 ± 0.2 ng ml^{-1} h^{-1} (n=12, P<0.05). Interestingly, in the STNx ewes on the high salt diet, the increase in urinary sodium output was greater than the increase in sodium intake, so their sodium balance became negative. Fetal plasma renin levels rose from 10 ± 7.7 (n=6) before salt, to 19.3 ± 7.4 ng ml^{-1} h^{-1} (n=6) after salt (P=0.05 after log transformation of the data). By contrast, in the control ewes on the high salt diet, maternal sodium balance remained positive, and there was no change in fetal plasma renin levels (before salt 11.0 ± 5.2, n=5; after salt 12.8 ± 8.8 ng ml^{-1} h^{-1}, n=5).

It is concluded that the fetal renin angiotensin system was suppressed in this model of maternal renal dysfunction. The renin angiotensin system is essential for normal renal development (Guron & Friberg, 2000). Therefore, by suppressing this system, maternal renal dysfunction may impair fetal renal development and predispose the offspring to hypertension. Furthermore, fetuses whose mothers have renal impairment may be exposed to greater fluctuations in salt and water balance than those whose mothers have normal renal function.

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The role of renal sympathetic nerve activity in the hypertension induced by chronic nitric oxide blockade

R. Ramchandra, C.J. Barrett, S.J. Guild and S.C. Malpas, Circulatory Control Lab, Department of Physiology, University of Auckland, Private Bag 92019, Auckland, New Zealand.

Chronic blockade of nitric oxide leads to an increase in blood pressure that is maintained over the period of the blockade in baroreceptor intact animals (Scrogin *et al.*, 1998). In addition to the vascular actions of endothelium-derived nitric oxide, indirect evidence supports a role for the sympathetic nervous system in maintaining the hypertension. The decrease in blood pressure with ganglionic blockade is exaggerated with blockade of nitric oxide suggesting an increase in sympathetic tone (Liu *et al.*, 1998; Scrogin *et al.*, 1998). Guanethidine-induced sympathetectomy also attenuates the hypertension induced by chronic nitric oxide blockade (Sander *et al.*, 1997).

In order to test this possible interaction between nitric oxide and sympathetic nerve activity directly, we measured arterial pressure and renal sympathetic nerve activity before, during and after nitric oxide blockade using L-NAME (50mg/kg/day *via* drinking water) over 7 days, in baroreceptor intact and sino-aortic denervated (SAD) conscious rabbits.

In the baroreceptor intact animals, blockade of nitric oxide led to a signifi cant increase in mean arterial pressure (from 75 ± 2 to 84 ± 3 mmHg) and decrease in heart rate (from 233 ± 8 to 195 ± 8 bpm) that was sustained over the 7 days of nitric oxide blockade. In all SAD animals, an initial increase in arterial pressure (82 ± 3 mmHg on the second day) was seen but was not sustained and recovered back to pre L-NAME levels. Direct recordings of renal sympathetic nerve activity suggest the increase in blood pressure in the baroreceptor intact animals is not accompanied by a change in renal sympathetic tone (9 ± 3 normalised units during control v/s 10 ± 4 normalised units at day 7 of L-NAME treatment). There is evidence of resetting of the blood pressure- renal sympathetic nerve activity baroreflex curve such that blood pressure is maintained at a hypertensive level.

In summary, our results do not support a role for increased renal sympathetic nerve activity in maintaining the hypertension with nitric oxide blockade in baroreceptor intact animals. The lack of a sustained increase in pressure in the SAD animals suggests an important role for baroreflexes in the long-term control of arterial pressure.

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Baroreflexes play a major role in regulating the long-term level of sympathetic nerve activity

S.J. Guild, C.J. Barrett, R. Ramchandra and S.C. Malpas, Circulatory Control Laboratory, Department of Physiology, University of Auckland, Private Bag 921019, Auckland, New Zealand.

It is accepted dogma that arterial baroreflexes play no role in the long-term regulation of arterial pressure because they exhibit resetting in response to sustained increases in arterial pressure. However, a recent study from our laboratory (Barrett *et al.*, 2003) challenges this view. We observed that chronic infusion of angiotensin II (7 days, 50ng/kg/min i.v.) caused an increase in mean arterial pressure (MAP) and a sustained decrease in renal sympathetic nerve activity (RSNA). Also, although the MAP-heart rate baroreflex curve showed resetting, the MAP-RSNA baroreflex curve did not. These results suggest that baroreflex control of RSNA is likely to play a significant role in the long-term control of arterial pressure. Full interpretation of these results is however made difficult by the direct and central nervous system effects of angiotensin II on RSNA. It is important therefore to investigate this result further using an alternative method of increasing arterial pressure. In this current study chronic infusion of phenylephrine was chosen.

In New Zealand white rabbits, living in their home cages, arterial pressure and RSNA were recorded continuously using telemetry devices before, during and after a 7-day infusion of phenylephrine (30mg/kg/hr i.v.) using an osmotic mini pump. The modest but sustained increase in MAP during phenylephrine infusion was accompanied by significant bradycardia and decreased RSNA (˜30%) over the 7-day infusion period. Baroreflex responses were derived using rapid infusions of sodium nitroprusside and phenylephrine before, at day 2, and 7 of phenylephrine infusion and again after removal of the osmotic pump. The MAP-RSNA curves during phenylephrine infusion not only showed no evidence of the rightward shift suggesting resetting, but also showed a decrease in range and the resting points lie near the lower plateau of these curves suggesting that the decreased RSNA observed during phenylephrine infusion is due to the baroreflex. These results suggest that the similar changes to the baroreflex curves observed during angiotensin II infusion are independent of the central or direct effects of angtiotensin II and are mediated by the inducement of hypertension. Overall these results support the notion that the baroreflexes **do** play an important role in regulating the long-term level of RSNA.

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NO and α-adrenoceptor subtypes in regional renal vascular responses to renal nerve stimulation in rabbits

G.A. Eppel[1], L.L. Lee[1], K.M. Denton[1], S.C. Malpas[2] and R.G. Evans[1], [1]Department of Physiology, Building 13F, Monash University, VIC 3800, Australia and [2]Department of Physiology, University of Auckland, Auckland, New Zealand.

Introduction. Renal medullary blood flow (MBF) plays a critical role in long-term control of arterial pressure. Therefore understanding the mechanisms controlling MBF is important. When renal sympathetic drive is increased reflexively (Leonard *et al.*, 2001), or by electrical stimulation (RNS, Leonard *et al.*, 2000), MBF is reduced much less than cortical or total renal blood flow (CBF, RBF). Zou and Cowley (2000) have shown that MBF responses to noradrenaline infusion are blunted by α_2-adrenoceptor mediated NO release. We tested whether this mechanism blunts MBF responses to RNS in pentobarbitone anaesthetised rabbits (90-150mg + 30-50mg/h iv).

Methods. RBF was measured by transit-time ultrasound flowmetry, CBF and MBF were measured by laser-Doppler flowmetry. RNS was performed at a supramaximal voltage (2ms pulse duration) for 3min at each frequency (0.5,1,2,4 and 8Hz in random order). In Experiment 1, RNS was performed before and after prazosin (α_1-adrenoceptor antagonist; 0.2mg/kg + 0.2mg/kg/h iv; n=6), rauwolscine (α_2-adrenoceptor antagonist; 0.5mg/kg + 0.25mg/kg/h iv; n=6) or vehicle treatment (n=6). In Experiment 2, responses to RNS were measured under control conditions, after NO synthase blockade with N^G-nitro-L-arginine (L-NNA, 20mg/kg/min + 5mg/kg/h iv), and then during co-infusion of glyceryl trinitrate at a dose that restored arterial pressure and RBF to control levels (10-50 μg/kg/min iv). A second group (n=6) served as a time control, receiving only vehicle treatment.

Results. In all groups RBF, CBF and to a lesser extent MBF, were reduced by RNS in a stimulus-dependent manner. In Experiment 1, prazosin decreased baseline arterial pressure by −11±4% and CBF by −18±3%. Prazosin blunted RNS-induced responses of RBF and CBF but not MBF. For example at 4Hz, RBF, CBF and MBF were reduced by −85±3%, −89±2% and −20±12% respectively during the control period, and by −39±3%, −42±5% and −28±7% during prazosin treatment. Rauwolscine increased arterial pressure by 8±2% and decreased RBF by −25±2% and CBF by −14±3%. Rauwolscine increased CBF and MBF responses to RNS but only at frequencies ≤ 2Hz. For example RNS at 1Hz reduced CBF by −21±2% but not MBF (+9±9%) during the control period. During rauwolscine treatment, RNS at 1Hz reduced CBF by −30±6% and MBF by −12±8%. Baseline haemodynamic variables and responses to RNS were not significantly affected by vehicle treatment. In Experiment 2, L-NNA increased arterial pressure by 34±4% and decreased RBF and MBF by −16±2% and −52±5% respectively. L-NNA treatment enhanced responses to RNS, particularly MBF at the lower frequencies. For example, stimulation at 2Hz during the control period reduced RBF by −48±7% and CBF by −39±6% but not MBF (+1±18%). During L-NNA treatment the responses were −58±6%, −43±4% and −32±11% for RBF, CBF and MBF respectively. Glyceryl trinitrate infusion restored arterial pressure, RBF and MBF to control levels and also restored RBF, CBF and MBF responses to RNS to their control levels. Responses to RNS remained relatively stable in the time control group.

Conclusions. These data indicate that both α_2-adrenoceptor activation and NO blunt MBF responses to RNS at low frequencies, but also blunt CBF responses to some extent. Whether the impact of α_2-adrenoceptor activation is mediated by NO, remains to be determined. MBF remained less responsive to RNS than CBF during NO synthase and α_2-adrenoceptor blockade, indicating that other mechanisms also contribute to the differential impact of RNS on CBF and MBF. α_1-adrenoceptors make an important contribution to RNS-induced changes in CBF but seem to contribute less to RNS-induced changes in MBF.

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Do nitric oxide and prostaglandins protect the renal medullary circulation from ischaemia during renal nerve stimulation?

N.W. Rajapakse[1], G.A. Eppel[1], K.M. Denton[1], S.C. Malpas[2] and R.G. Evans[1], [1]Department of Physiology, Monash University, Melbourne, Australia and [2]Department of Physiology, University of Auckland, Auckland, New Zealand.

Renal medullary blood flow (MBF) is less sensitive than cortical blood flow (CBF) to sympathetic activation (Guild *et al.*, 2002), in part because of a counter regulatory vasodilator role of nitric oxide (NO) (Eppel *et al.*, in press). Thus, blockade of NO-synthase in anaesthetised rabbits enhances responses of total renal blood flow (RBF), CBF, and particularly MBF, to renal nerve stimulation (RNS) (Eppel *et al.*, in press). However, other mechanisms must also be involved, because even after NO-synthase blockade, RNS still reduces CBF more than MBF.

In the present study we tested whether prostaglandins contribute to the relative insensitivity of MBF to renal sympathetic drive in pentobarbitone (90-150 mg + 30-50 mg h^{-1}) anaesthetised rabbits. We also tested the effects of NO-synthase inhibition on regional kidney blood flow responses to RNS in rabbits pre-treated with a cyclooxygenase inhibitor.

A transonic flow probe was used to measure RBF and laser-Doppler flow probes were used to measure CBF and MBF. Responses to RNS were tested before and after intravenous ibuprofen (12.5 mg/kg plus 12.5 mg/kg/h; n = 18) or its vehicle (n = 6). In ibuprofen-treated rabbits, responses were then tested after N^G-nitro-L-arginine (L-NNA; 20 mg/kg + 5 mg/kg/h; n=6), L-NNA + glyceryl trinitrate (GTN; 8 - 22 µg/kg/min; n = 6) or vehicle (n = 6).

Ibuprofen but not its vehicle reduced basal RBF, CBF and MBF. Subsequent treatment with L-NNA, but not L-NNA + GTN or vehicle, increased mean arterial pressure and reduced RBF and MBF. RNS (0.75 – 6 Hz) caused stimulus-dependent reductions in RBF (85 ± 4% at 6 Hz) and CBF (87 ± 3% at 6 Hz) more than MBF (36 ± 14% at 6 Hz) in vehicle-treated rabbits. Ibuprofen did not significantly affect responses of RBF, CBF or MBF to RNS. L-NNA, but not vehicle or L-NNA + GTN, significantly enhanced RNS-induced reductions in RBF ($P \leq 0.001$) and CBF ($P = 0.02$) but not MBF ($P = 0.8$).

We conclude that cyclooxygenase products have little net impact on regional kidney blood flow responses to RNS. Our finding that NOS blockade did not affect responses of MBF to RNS after cyclooxygenase blockade contrast with our previous findings in rabbits with intact cyclooxygenase activity (Eppel *et al.*, in press). This may reflect interactions between nitric oxide and vasoconstrictor prostaglandins in modulating responses of MBF to RNS. This notion is consistent with previous studies of isolated perfused kidneys, in which NO blockade enhances vasoconstrictor responses to noradrenaline under control conditions, but not after cyclooxygenase blockade (Zhang & Sassard, 1993).

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