Fetal endocrine responses to prolonged reduced uterine blood flow are altered following bilateral sectioning of the carotid sinus and vagus nerves

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Abstract

The present study examines the effect of carotid sinus/vagosympathetic denervation on fetal endocrine responses to prolonged reduced uterine blood flow (RUBF). Fetal sheep had vascular catheters inserted following bilateral sectioning of the carotid sinus and vagus nerves (denervated, $n=7$) or sham denervation (intact, $n=7$). Uterine blood flow was mechanically restricted at 126.1 ± 0.7 days (mean $\pm$ S.E.M.) for 24 h, decreasing arterial oxygen saturation by $47.3 \pm 2.6\%$ ($P<0.01$). Fetal plasma samples were obtained at -1, 3, 6, 12 and 24 h for subsequent analyses of arginine vasopressin (AVP), angiotensin II and catecholamines. The AVP response to prolonged RUBF was markedly attenuated in denervated fetuses (15.6 ± 3.6 to 34.9 ± 6.0 pg/ml) when compared with intact (10.0 ± 1.4 to 127.3 ± 28.4 pg/ml). In contrast, intact

fetuses demonstrated no change in plasma angiotensin II concentrations with RUBF whereas denervated fetuses demonstrated a marked increase from 47.5 ± 18.9 to 128.7 ± 34.2 pg/ml. The norepinephrine and epinephrine responses to prolonged RUBF were attenuated in denervated fetuses (950.1 ± 308.9 and 155.8 ± 58.5 to 1268.3 ± 474.6 and 290.6 ± 160.2 pg/ml respectively) when compared with intact (1558.3 ± 384.4 and 547.3 ± 304.7 pg/ml to 3289.2 ± 1219.8 and 896.8 ± 467.8 pg/ml respectively). These results support a role for the peripheral chemoreceptors in mediating fetal endocrine responses to prolonged RUBF, which may in part lead to the altered cardiovascular responses observed in denervated fetuses under these conditions.

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Introduction

Under conditions of acute hypoxia in sheep, fetal plasma arginine vasopressin (AVP) (Rurak 1978, Daniel *et al.* 1983), angiotensin II (Broughton Pipkin *et al.* 1974) and catecholamine (Jones & Robinson 1975, Gu *et al.* 1985) concentrations increase. The role of the peripheral chemoreceptors in mediating these responses remains controversial. Sectioning of the cervical vagosympathetic trunk attenuates the rise in plasma AVP concentrations (Rurak 1978) whereas sinoaortic (Raff *et al.* 1991) or carotid sinus denervation (Giussani *et al.* 1994) does not. Furthermore, sinoaortic denervation attenuates the rise in plasma angiotensin II concentrations (Wood *et al.* 1990) whereas carotid sinus denervation does not (Green *et al.* 1997). The role of the peripheral chemoreceptors in mediating the rise in plasma catecholamine concentrations during acute hypoxia has not been examined, although Jensen & Hanson (1995) have reported an attenuation of the catecholamine

response to acute fetal asphyxia following carotid sinus denervation.

When hypoxia is maintained, plasma AVP concentrations return to normoxic values after 12 h whereas plasma catecholamine concentrations remain elevated (Hooper *et al.* 1990). The fetal angiotensin II response to prolonged hypoxia has not been characterized. The role of the peripheral chemoreceptors in mediating fetal endocrine responses to prolonged hypoxia has also not been studied. Furthermore, the contribution of fetal endocrine responses to the sustained tachycardia and transient rise in arterial blood pressure observed during prolonged hypoxia is not known (Bocking *et al.* 1988). Recent studies in our laboratory, however, suggest a role for the peripheral chemoreceptors in mediating these cardiovascular alterations during prolonged reduced uterine blood flow (RUBF) (Stein *et al.* 1997).

These studies were designed to test the hypothesis that fetal endocrine responses to prolonged hypoxia are mediated in part by peripheral chemoreceptor function.

Fetal plasma AVP, angiotensin II and catecholamine concentrations were therefore measured before and during prolonged fetal hypoxia, secondary to RUBF, in intact and carotid sinus/vagovagal denervated fetal sheep.

Materials and Methods

Surgical procedures

Surgery was performed on 14 pregnant sheep of known mating dates between 118 and 126 days gestation under general anaesthesia (intravenous thiopental sodium for induction; 1.0–1.5% halothane in oxygen for maintenance). A polyvinyl catheter was placed in a maternal femoral vein (V11; Bolab, Lake Havasu City, AZ, USA) followed by a vascular clamp around the maternal common internal iliac artery. Carotid sinus denervation was performed bilaterally in seven fetuses followed by bilateral mid-cervical vagotomy; these were termed 'denervated' fetuses. In seven fetuses serving as controls, these nerves were identified and left uncut; these were termed 'intact' fetuses. Catheters were placed in the fetal carotid and brachiocephalic arteries, jugular vein, trachea (V4; Bolab) and amniotic cavity (V11; Bolab). A transit-time flow transducer (Transonic Inc, Ithaca, NY, USA) was placed around the contralateral carotid artery. All cables, catheters and electrodes were exteriorized through the maternal flank. Animals were housed in individual cages with free access to food and water, and allowed 4 days to recover from surgery before experiments were commenced. All animals were treated in compliance with guidelines established by the Canadian Council on Animal Care and according to protocols approved by the Animal Care Committees of the Lawson Research Institute and the University of Western Ontario.

Experimental protocol

All experiments began between 0900 and 1000 h with a 2 h control period. At time 0, the vascular clamp was adjusted such that uterine blood flow was reduced sufficiently to decrease fetal arterial oxygen saturation (SaO_2) by approximately 50%. Fetal arterial blood samples (0.2 ml) were drawn at – 5, 5, 10 and 15 min and 1, 2, 3, 6, 12, 16, 20 and 24 h to ensure a stable reduction in fetal SaO_2 . Blood gases and pH were determined at these time intervals using an ABL blood gas analyzer (Radiometer, Copenhagen, Denmark). Additional arterial blood samples (3.0 ml) were drawn at – 1, 3, 6, 12 and 24 h for subsequent measurement of plasma AVP, angiotensin II and catecholamine concentrations. Fetal blood samples (2.0 ml) used for the determination of plasma AVP and angiotensin II concentrations were transferred to glass tubes whereas blood samples (1.0 ml) used for the determination of plasma catecholamine concentrations were

immediately transferred to glass tubes containing sodium metabisulphite/EDTA. All tubes were centrifuged at 2800 g for 5 min at 4 °C. Plasma was then removed and stored at – 80 °C for subsequent analyses.

Plasma AVP concentrations

Plasma AVP concentrations were measured as previously described (Giussani *et al.* 1996) after chromatographic separation using a double-antibody RIA with reagents purchased as a kit (Mitsubishi Yuka, Mitsubishi Petrochemical Co. Ltd, Japan, distributed in the UK by IDS Ltd, Boldon, Tyne and Wear, UK). SepPak C_{18} cartridges (Waters Associates, Milford, MA, USA) were mounted on a Super Separator-24 manifold and washed with 10 ml volumes of methanol and distilled water. Plasma samples (0.5 ml) were mixed with equal volumes of 0.1 M HCl and applied to columns. Columns were washed with 10.0 ml 4% acetic acid, and AVP was eluted with 2.5 ml methanol. Extracts were dried at 37 °C with a jet of air, and residues were reconstituted in 1.0 ml phosphate buffer. Duplicate aliquots (volume dependent on expected AVP concentration) were adjusted to 0.3 ml with phosphate buffer, and AVP antiserum was added (0.1 ml). Samples were mixed and incubated at 4 °C for 20 h. Then 0.1 ml ^{125}I -AVP was added, and tubes were incubated at 4 °C for an additional 20 h. A second antibody (0.1 ml) and polyethylene glycol (0.4 ml) were added, and samples incubated at 4 °C for 4 h. Samples were centrifuged (2000 g) at 4 °C for 30 min and the supernatants decanted. The remaining residue was then counted for radioactive content. The interassay coefficients of variation for two plasma samples (2.71 pg/ml and 5.55 pg/ml AVP) were 4.1 and 9.8% respectively.

Plasma angiotensin II concentrations

Plasma angiotensin II concentrations were measured as previously described (Giussani *et al.* 1996) after chromatographic separation using a sensitive and specific competitive protein-binding RIA with reagents supplied as a kit (Nichols Institute, Diagnostics BV, Saffron Walden, Essex, UK). Plasma samples (0.5 ml) were mixed with equal volumes of 10 mmol/l phosphate buffer (pH 7.4), and columns (SepPak C_{18}) washed with 10 ml volumes of methanol and phosphate buffer. Samples were then applied to the columns and washed with 10 ml phosphate buffer. Angiotensin II was eluted with 2.5 ml ethanol, and extracts were dried at 37 °C with a jet of air. Residues were reconstituted in 0.84 ml Tris buffer, and duplicate aliquots (0.4 ml) transferred to polystyrene tubes. A 0.1 ml volume of anti-angiotensin II (rabbit) antiserum was added and samples mixed and incubated at 2–8 °C for 6 h. Samples were treated with 0.1 ml ^{125}I -angiotensin II and incubated at 4 °C for 18 h. Anti-rabbit antiserum (donkey) precipitant (0.1 ml) was added, and tubes were incubated

at room temperature for 30 min. A 1.0 ml volume of deionized water was added and tubes were centrifuged (2000 g) for 15 min at room temperature. The supernatant was then decanted and remaining residue counted for radioactive content. The sensitivity of the assay was 3.8 pg/ml. The intra- and inter-assay coefficients of variation for three plasma samples (31, 42 and 96 pg/ml) were 5.1, 4.0 and 9.3% respectively.

Plasma catecholamine concentrations

Epinephrine and norepinephrine were measured by electrochemical detection after HPLC fractionation. Fetal plasma samples (0.4 ml) were spiked with 50 µl of the internal standard 6-methyldopamine after microcentrifugation for 1 min and treated with 400 µl 200 mmol/l Tris-EDTA. Samples were allowed to equilibrate for 5 min and then subjected to solid-phase extraction using 3.0 ml 200 mg Alumina A mini-columns (J & W Scientific Inc., Folsom, CA, USA) to adsorb all catecholamines. Columns were washed four times with 4 mmol/l Tris-EDTA at pH 8.5, and catecholamines were eluted with 2.0 ml glacial acetic acid at pH 3.0. Extracts were dried using a Speedvac Concentrator (Savant) and the residue reconstituted in 150 µl HPLC eluent and subjected to reverse-phase HPLC on a Bondapak column (30 × 0.39 cm; Waters, Mississauga, Ontario, Canada). Catecholamines were eluted with buffer consisting of 50 mmol/l sodium acetate, 20 mmol/l citric acid, 0.135 mmol/l sodium EDTA, 1.0 mmol/l di-*n*-butylamine in water/methanol (95:5, v/v) at pH 4.3 (containing 3.75 mmol/l sodium-1-octanesulphonate as an ion-pairing reagent) at a flow rate of 1.0 ml/min. The column eluent was monitored with a model M460 electrochemical detector (Waters) in the oxidative mode at a potential difference of 0.6 V. The peaks were quantified with a model M740 data module (Waters) and compared with those of authentic reference standards of epinephrine, norepinephrine and 6-methyldopamine.

Statistical analyses

All results are presented as mean values ± S.E.M. Statistical significance was determined using a two-way ANOVA with repeated measures (BMDP 5 V; BMDP Statistical Software Inc., Los Angeles, CA, USA) comparing the effect of time and group. If a significant effect of time or group was found ($P < 0.05$), within-animal comparisons were conducted using Dunnett's post-hoc test (BMDP 7D) and between-group comparisons were made using Student's unpaired *t*-test.

Results

Blood gases and arterial oxygen saturation

During the normoxic control period, fetal blood gases and SaO_2 were similar in both groups. After the onset of

RUBF, SaO_2 decreased ($P < 0.01$) similarly in intact and denervated fetuses (62.4 ± 5.0 and $60.3 \pm 6.1\%$ to 35.4 ± 2.7 and $28.2 \pm 2.0\%$ respectively) whereas arterial pCO_2 did not change (50.9 ± 0.8 and 51.3 ± 1.6 mmHg to 51.4 ± 1.8 and 55.9 ± 3.5 mmHg respectively). Arterial pH decreased significantly at 1 h in both intact and denervated fetuses (7.36 ± 0.01 and 7.38 ± 0.01 to 7.28 ± 0.02 and 7.25 ± 0.02 respectively) as a result of a transient decrease in base excess followed by a return towards control values at 12 h. At 20 and 24 h, arterial pH was significantly lower (7.26 ± 0.03 and 7.27 ± 0.05 respectively; $P < 0.05$) in denervated fetuses when compared with the normoxia period, although not significantly different from intact fetuses.

Plasma AVP concentrations

Under normoxic conditions, fetal plasma AVP concentrations were similar in intact and denervated animals. After the onset of RUBF, plasma AVP concentrations increased significantly ($P < 0.01$) in intact fetuses from 10.0 ± 1.4 pg/ml to a maximum value of 127.3 ± 28.4 pg/ml at 6 h and this was followed by a return to normoxic values (Fig. 1). In denervated fetuses, the AVP response was markedly attenuated, increasing from 15.6 ± 3.6 pg/ml to a maximum value of only 34.9 ± 6.0 pg/ml ($P < 0.05$) at 6 h followed by a return to normoxic values.

Plasma angiotensin II concentrations

Under normoxic conditions, plasma angiotensin II concentrations were similar in intact and denervated fetuses. Plasma angiotensin II concentrations did not change in intact fetuses with the onset of RUBF whereas denervated fetuses demonstrated a marked increase from 47.5 ± 18.9 pg/ml to a maximum value of 128.7 ± 34.2 pg/ml at 12 h, followed by a return to normoxic values (Fig. 2). Plasma angiotensin II concentrations were significantly greater ($P < 0.05$) in denervated fetuses at 3, 6 and 12 h of RUBF when compared with intact fetuses.

Plasma catecholamine concentrations

Under normoxic conditions, plasma norepinephrine and epinephrine concentrations were similar in intact and denervated fetuses. With the onset of RUBF, plasma norepinephrine concentrations increased in intact fetuses from 1558.3 ± 384.4 pg/ml to a maximum value of 3289.2 ± 1219.8 pg/ml at 12 h, although this was not statistically significant, and remained elevated throughout the 24 h RUBF period (Fig. 3A). In contrast, denervated fetuses demonstrated no change in plasma norepinephrine concentrations. Plasma epinephrine concentrations demonstrated a modest non-significant increase in intact

Figure 1 Plasma AVP concentrations measured at selected intervals before and during the 24 h RUBF period in intact (○) and denervated (●) fetuses. Values are means $\pm$ S.E.M. * $P < 0.01$, § $P < 0.05$, values significantly different from pre-RUBF values. † $P < 0.05$, †† $P < 0.01$, values significantly different from those for denervated fetuses. Bar represents period of RUBF.

fetuses from 547.3 ± 304.7 pg/ml to a maximum value of 896.8 ± 467.8 pg/ml at 6 h, whereas denervated fetuses demonstrated no change (Fig. 3B).

Discussion

In the present study we have shown that the AVP and catecholamine responses to prolonged RUBF are markedly attenuated following peripheral chemodenervation. In addition, plasma angiotensin II concentrations increase in denervated fetuses in response to prolonged RUBF whereas intact fetuses demonstrate no change. We propose that alterations in the release of vasoactive hormones in denervated fetuses are responsible in part for the altered cardiovascular responses to prolonged RUBF observed in these animals (Stein *et al.* 1997).

AVP is known to be an important mediator of fetal cardiovascular responses to acute hypoxia. AVP administration to normoxic fetuses leads to cardiovascular changes similar to those observed with acute hypoxia including a transient bradycardia and hypertension (Rurak 1978, Iwamoto *et al.* 1979, Tomita *et al.* 1985). Furthermore, fetal plasma AVP concentrations increase with the onset of acute hypoxia (Rurak 1978, Daniel *et al.* 1983, Raff *et al.* 1991), and administration of a V_1 receptor antagonist partially reverses the associated cardiovascular

responses (Perez *et al.* 1989). The role of the peripheral chemoreceptors in mediating the AVP response to acute hypoxia remains controversial. Bilateral sectioning of the cervical vagosympathetic trunk attenuates the response (Rurak 1978), whereas sinoaortic (Raff *et al.* 1991) or carotid sinus (Giussani *et al.* 1994) denervation does not. During prolonged RUBF, fetal plasma AVP concentrations increase to maximum values at 2 h and return to normoxic values after 12 h (Hooper *et al.* 1990), suggesting a transient role in modulating fetal cardiovascular responses. The current study is unique in that it examines for the first time the role of the peripheral chemoreceptors in mediating the fetal AVP response to prolonged hypoxia. In sectioning the carotid sinus and vagus nerves, afferent fibres from sensory receptors other than the carotid and aortic chemoreceptors are removed. It is therefore possible that vagal afferents, including those of low-pressure baroreceptors in the atrium, stimulate the AVP response.

Fetal plasma AVP concentrations increase in response to other stimuli including elevated plasma osmolality and adenosine concentrations (Kelly *et al.* 1983, Ross *et al.* 1992). A direct measure of plasma osmolality was not available in the current study, although fetal hematocrit did not change throughout the RUBF period and was similar in both groups. Adenosine, a metabolic product of oxygen deficiency, also increases with acute hypoxia (Koos & Doany 1991). Adenosine infusion increases plasma AVP

Figure 2 Plasma angiotensin II concentrations measured at selected intervals before and during the 24 h RUBF period in intact (○) and denervated (●) fetuses. Values are means $\pm$ S.E.M. † $P < 0.05$, values significantly different from those for intact fetuses. Bar represents period of RUBF.

concentrations in fetal sheep whereas infusion of an adenosine receptor antagonist attenuates the rise in plasma AVP concentrations during acute hypoxia (Koos *et al.* 1994). It would thus be of interest to determine the effect of peripheral chemodenervation on fetal adenosine concentrations during prolonged RUBF.

The sustained tachycardia observed in intact fetuses with prolonged RUBF has previously been demonstrated in our laboratory (Bocking *et al.* 1988) and is thought to be secondary to β -adrenergic stimulation associated with a sustained elevation in plasma catecholamine concentrations (Hooper *et al.* 1990, Bocking *et al.* 1995). Jensen & Hanson (1995) reported that the increase in catecholamine levels associated with acute fetal asphyxia is delayed following carotid chemodenervation, suggesting that sympathetically mediated catecholamine release is in part a carotid chemoreflex. The lower circulating catecholamine levels observed in denervated fetuses in the present study would support an additional role for the peripheral chemoreceptors in mediating adrenomedullary catecholamine release under conditions of prolonged RUBF.

The lower circulating catecholamine levels observed in denervated fetuses may reflect changes in the neural control of catecholamine release mediated through sympathetic efferent innervation of the adrenal gland. Alternatively, changes in adrenal blood flow related to both splanchnic nerve activity and changes in local factors affecting adrenomedullary capillary dilatation may account for this decrease. Fetal adrenal blood flow in both the

medullary and cortical regions decreases in response to acute hypoxia following splanchnicotomy (Buchwalder *et al.* 1996). Furthermore, a study in adult dogs has demonstrated an increase in adrenal medullary blood flow in response to splanchnic nerve stimulation (Breslow *et al.* 1993). After administration of the nitric oxide (NO) synthase inhibitor L-NAME, however, splanchnic nerve stimulation no longer had an effect on medullary blood flow, suggesting that the increase is mediated through increased NO levels. It would be of interest to determine the role of NO in mediating adrenal blood flow changes in the fetus in response to both acute and prolonged hypoxia.

The fetal renin-angiotensin system responds to various stimuli, including acute hypoxia, with increases in both plasma renin activity and angiotensin II concentrations (Broughton Pipkin *et al.* 1974, Robillard *et al.* 1981, 1984, Tomita *et al.* 1985). Angiotensin II administration to normoxic fetuses alters heart rate and blood flow through selected vascular beds, and increases arterial blood pressure (Lumbers & Reid 1978, Iwamoto & Rudolph 1981). Green *et al.* (1997) have demonstrated, however, that fetal cardiovascular responses to acute hypoxia are not dependent on angiotensin II. In contrast, angiotensin II does appear to modulate arterial blood pressure and femoral vascular tone during acute hypoxia following carotid sinus denervation, as captopril infusion attenuates the rise in arterial pressure and femoral vascular resistance under these conditions (Green *et al.* 1997). In the current study, plasma angiotensin II concentrations did not change in

Figure 3 Plasma norepinephrine (A) and epinephrine (B) concentrations measured at selected intervals before and during the 24 h RUBF period in intact (○) and denervated (●) fetuses. Values are means $\pm$ S.E.M. Bar represents period of RUBF.

intact fetuses throughout the 24 h RUBF period, suggesting that angiotensin II does not normally mediate cardiovascular responses to prolonged hypoxia. It is of note, however, that the first measurement of angiotensin II in the present study, under RUBF conditions, was at 3 h. We therefore cannot exclude a possible earlier rise in plasma angiotensin II concentrations in intact fetuses. In denervated fetuses, however, the angiotensin II response to prolonged RUBF was markedly different, increasing to values significantly greater than in intact fetuses by 3 h and returning to normoxic values by 24 h. These observations are consistent with the proposed role for angiotensin II in regulating fetal cardiovascular responses to hypoxia once peripheral chemoreflex mechanisms are removed (Green *et al.* 1997).

The rise in plasma angiotensin II concentrations in denervated fetuses may also occur in response to the sustained hypotension observed in these animals. A decrease in arterial blood pressure stimulates renin secretion via intra- and extra-renal baroreceptor mechanisms, subsequently increasing plasma angiotensin II concentrations. Alternatively, peripheral chemodenervation may have affected angiotensin II clearance which occurs largely

in the fetal placental vascular bed (Lumbers & Reid 1978, Rosenfeld *et al.* 1995). Placental blood flow is significantly reduced during acute hypoxia in both vagotomized and sinoaortic denervated fetuses (Jansen *et al.* 1989) and it is therefore possible that reduced placental blood flow in peripheral chemodenervated fetuses caused a decrease in angiotensin II clearance with prolonged RUBF.

In summary, we have shown for the first time that increases in plasma AVP and catecholamine concentrations during prolonged RUBF are attenuated following peripheral chemodenervation in fetal sheep. These alterations in the AVP and catecholamine responses to prolonged RUBF may account for the altered cardiovascular responses observed in denervated fetuses. In addition, fetal plasma angiotensin II concentrations increase during prolonged RUBF following bilateral sectioning of the carotid sinus and vagus nerves, providing evidence that angiotensin II plays a secondary compensatory role in mediating fetal cardiovascular responses to prolonged RUBF once peripheral chemoreceptor mechanisms are removed.

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