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Effects of hypoxia on plasma amino acids of fetal sheep

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Summary. Secondary amino acid disturbances from circulatory responses during hypoxia may cause problems in interpreting plasma amino acid profiles of sick babies investigated for possible inherited defects. Systematic studies to characterise them are difficult in man. We investigated the effects of hypoxia on plasma amino acids by studying 9 late gestation fetal sheep *in utero* during 11 one hour episodes of moderately severe isocapnic hypoxia. In 6 experiments, maternal plasma amino acids were also monitored. Fourteen fetal plasma amino acids increased significantly, with the largest proportionate changes in alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, ornithine and lysine. Maternal amino acids did not increase. Probable explanations were reflex peripheral vasoconstriction in skeletal muscle beds and decreased hepatic blood flow. The findings extend our knowledge of the fetal response to hypoxic stress, demonstrate the importance of skeletal muscle in branched-chain amino acid metabolism, and should help with interpretation of postnatal plasma amino acid disturbances.

Keywords: Amino acids – Plasma – Hypoxia – Fetus – Branched-chain amino acids

Introduction

In clinical laboratories, analysis of plasma and urine amino acids is requested most often to investigate the possibility of an inherited amino acid defect. Samples are frequently from very sick babies or young children who may be poorly perfused, dehydrated or hypoxic. It is sometimes difficult to decide whether abnormalities in an amino acid profile are due to a primary

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amino acid defect or are merely secondary disturbances due to illness. Misinterpretation may lead to unnecessary alarm or to an inborn error being missed. From careful clinical observation, many secondary disturbances in *urinary* amino acids have been identified and documented (Blom and Huijmans, 1985). There is still little published information for plasma amino acids. Systematic studies are not possible in sick babies and many coincident physiological disturbances and therapeutic interventions could contribute to altered amino acid metabolism. Findings in plasma from dead babies (Briddon and Oberholzer, 1986) may have been affected by post mortem proteolysis.

It was the purpose of this study, therefore, to examine the effects of hypoxia and peripheral circulatory insufficiency on plasma amino acids, as part of an investigation of the physiological responses of late gestation fetal sheep to moderately severe hypoxia *in utero*. The hypoxic insult causes only transient disturbances from which the fetuses recover completely. In an earlier study with this model, we demonstrated a disturbance of organic acid metabolism that was attributable to changes in peripheral blood flow (Walker et al., 1996) and was similar to that of human newborns with birth asphyxia (Walker and Mills, 1992). We predicted that these circulatory changes would also be reflected in the plasma amino acid profile. If confirmed, this would be relevant to amino acid metabolism in sick babies and should help in evaluation of abnormal amino acid profiles.

Methods

Fetal studies

All procedures were conducted under licence and in accordance with the Animals (Scientific Procedures) Act, UK, 1986. Details of the animal surgical preparation and maintenance have been described previously (Giussani et al., 1993; Walker et al., 1996). Briefly, 9 singleton fetal sheep (Suffolk and Blue-faced Leicester Mule Cross) were instrumented at 114–129 (median 121) days of gestation (term is 147 days) under general anaesthesia (1 g thiopentone intravenously (i.v.) for induction, then 1–2% halothane in oxygen for maintenance) using sterile techniques. Fetal catheters were placed in a carotid artery and jugular vein and the amniotic sac. Pairs of stainless-steel electrodes were sewn onto the chest and hind limb to record the fetal electrocardiogram (ECG), into the diaphragm for the recording of fetal breathing movements, and biparietally on the dura for electrocorticogram recordings. The animals studied were primarily involved in other research which involved slightly different additional instrumentation. In 8 fetuses, ultrasonic flow transducers were placed around one femoral artery. All catheters and electrodes were exteriorised through the maternal flank. Benzylpenicillin sodium (Crystapen, 600 mg) was administered into the amniotic sac prior to closure of the uterus. A maternal femoral vein and artery was catheterised and the ewe given 5 ml of Streptopen intramuscularly (i.m.). A period of 5 days post-operative recovery was allowed during which time antibiotics were administered daily to the ewe (600 mg Crystapen i.v. for 4 days and 80 mg gentamicin i.v. daily for the first 3 days) and fetal arterial blood taken for blood gas analysis for assessment of fetal health. Catheters were maintained patent by continuous infusion of heparinised saline (40 U/ml at 0.2 ml/h).

A total of eleven acute hypoxia experiments was carried out at 119–134 (median 126) days of gestation. Of these, three were conducted 2 h after the ewe had been given 12 mg of dexamethasone intramuscularly. Two fetuses underwent 2 experiments separated by a

24–48 hour recovery period in which physiological and blood gas parameters returned to basal values. The experiments followed a 4 hour protocol. After a control period of normoxia of 2 hours, fetal isocapnic hypoxia was induced for 1 hour by reducing the maternal inspired oxygen fraction to 0.09 (18 L/min of air, 22 L/min of nitrogen and 1.2 L/min of carbon dioxide) (Giussani et al., 1993; Walker et al., 1996). The ewes were then returned to normoxia, and monitoring continued for 1 hour. Maternal and fetal arterial blood samples (0.4 ml) were taken at 15 and 45 min of each hour for blood gas determination (BG model IL 1301, Instrumentation Laboratories, UK) and for glucose and lactate measurements (YSI model 2300, Yellow Springs, Ohio, USA). Blood samples (2 ml) collected into chilled EDTA tubes were taken at the same time for amino acid analysis. These tubes were centrifuged within 10–15 min at 4°C (3,000 rpm) for 15 min. Plasma was decanted and stored at minus 20°C. On completion of the experiments, the fetuses were killed by an intravenous barbiturate overdose to the ewe.

Amino acid analysis

Amino acids were analysed by cation exchange chromatography using an LKB Alpha Plus-II amino acid analyser (Pharmacia LKB Biochrom Ltd., Cambridge, UK) with lithium buffers (Pharmacia LKB) and ninhydrin colour reagent, and detection at 440 nm and 570 nm. Data was processed with a Varian GC computer Star system (Walton-on-Thames, Surrey, UK). 100 μl of plasma was mixed with 100 μl of sulphosalicylic acid (60 g/L) with norleucine as internal standard, the final concentration being 250 μmol/L. 40 μl of supernatant was analysed with a 2 h 44 minute programme. Standard amino acid mixtures (Sigma Chemicals, Poole, Dorset, UK) diluted to a final concentration of 250 μmol/L for each amino acid were used for calibration. Freshly prepared glutamine standard was added to the calibrant mixtures for each analytical batch. Between-batch imprecision (CV%) in our laboratory ranges from 2% to 7% for the measured amino acids, except methionine (12%).

Statistics

Wilcoxon's test for paired data was used for comparisons of values during hypoxia with those during the control period, and for comparison of paired fetal and maternal data.

Results

Fetal plasma amino acids were measured during 11 hypoxia experiments. For 6 of these, paired maternal samples were also available for analysis. Values and statistical comparisons for pH and blood gases, glucose and lactate before, during and after hypoxia are presented in Tables 1 and 2. Fetal PaO_2 was reduced to around half its basal value during hypoxia, $PaCO_2$ was maintained, lactic acid increased significantly ($P < 0.01$) and pH fell, although the fetuses were not severely acidotic (Table 1). Lactic acid increased and pH decreased further, 15 minutes after relief of hypoxia. Maternal PaO_2 was reduced significantly ($P < 0.05$) but this had no impact on arterial pH and was associated with a small significant increase in plasma lactate but only in the first hypoxia sample (15 min). Femoral arterial blood flow was monitored in 9 experiments (8 fetuses). Blood flow decreased significantly during hypoxia from a baseline value of 43 (34–55) ml/min (median and range) to 12 (8–20) ml/min after 45 min of hypoxia ($P < 0.001$). After 45 min of re-oxygenation, flow was 65 (45–71) ml/min, which was significantly higher than basal ($P < 0.01$).

Table 1. Fetal blood gases, pH, lactate and glucose [mean (SEM)]

	†Prehypoxia	Hypoxia		Posthypoxia
		15 min	45 min	15 min
†† PaO_2 kPa	3.01 (0.12)	1.55 (0.06)**	1.62 (0.06)**	3.24 (0.18)
†† $PaCO_2$ kPa	6.31 (0.20)	6.17 (0.22)	6.18 (0.26)	6.12 (0.16)
†† pH	7.36 (0.01)	7.32 (0.01)*	7.25 (0.02)**	7.21 (0.03)**
glucose mmol/L	0.92 (0.09)	1.19 (0.11)*	1.51 (0.18)*	1.55 (0.25)*
n	9	9	10	8
lactate mmol/L	1.06 (0.07)	2.77 (0.29)**	5.15 (0.49)**	6.55 (0.92)**
n	11	11	11	10

†15 min before hypoxia; ††Data for 11 experiments. For glucose and lactate, data are shown for the number (n) of experiments indicated. Difference from pre-hypoxia: *$P < 0.05$; **$P < 0.01$ (Wilcoxon's test).

Table 2. Maternal blood gases, pH, lactate and glucose [mean (SEM)]†

	††Prehypoxia	Hypoxia		Posthypoxia
		15 min	45 min	15 min
PaO_2 kPa	13.66 (0.65)	4.80 (0.22)*	4.89 (0.23)*	14.43 (0.27)
$PaCO_2$ kPa	4.29 (0.20)	4.38 (0.09)	4.19 (0.13)	4.38 (0.22)
pH	7.46 (0.01)	7.46 (0.01)	7.46 (0.01)	7.45 (0.01)
glucose mmol/L	2.53 (0.21)	2.62 (0.27)	2.77 (0.35)	3.00 (0.45)
lactate mmol/L	0.19 (0.02)	0.33 (0.14)*	0.21 (0.07)	0.22 (0.08)
n	6	6	6	6

†Data for 6 experiments in which paired maternal and fetal plasma amino acids were analysed; ††15 min before hypoxia. Difference from prehypoxia: *$P < 0.05$.

Dexamethasone did not appear to alter the femoral blood flow response to hypoxia or the post-hypoxic rise in flow. All the fetuses recovered after the challenge, as indicated from physiological monitoring.

Plasma amino acids

Dexamethasone had no effect on the measurements and therefore results for all 11 fetal experiments were pooled (Table 3). After 45 min of hypoxia, concentrations of 14 of the 19 amino acids measured were more than 10% higher than basal control values (Fig. 1). The increases were statistically significant. Percentage increases were largest for alanine, the branched chain amino acids, lysine, tyrosine, phenylalanine and ornithine. Fifteen minutes after relief of hypoxia, plasma alanine increased further, and concentrations of thirteen other amino acids were still significantly higher than control values.

Table 3. Fetal plasma amino acid concentrations before, during and after hypoxia

	Plasma amino acid concentration (μmol/L)		
	† Prehypoxia mean (SEM)	45 min of Hypoxia mean (SEM)	15 min Posthypoxia mean (SEM)
Tau	64 (11)	81 (11)*	97 (20)*
Asp	67 (12)	70 (13)	69 (14)
Thre	201 (25)	235 (24)*	234 (24)*
Ser	812 (146)	798 (136)	835 (139)
Glu	90 (14)	91 (12)	90 (13)
Glmn	483 (29)	606 (60)*	615 (66)*
Gly	755 (88)	793 (91)	819 (94)
Ala	201 (12)	363 (29)**	439 (38)**
Cit	120 (7)	120 (6)	130 (6)
αAIB	41 (9)	42 (9)	45 (10)
Val	302 (25)	404 (36)**	417 (40)**
Meth	54 (12)	59 (12)**	60 (12)**
Isoleu	95 (7)	157 (17)**	163 (19)**
Leu	119 (11)	216 (23)**	227 (29)**
Tyr	78 (10)	105 (12)*	100 (14)**
Phe	101 (7)	140 (10)**	128 (8)**
Orn	60 (4)	79 (7)**	78 (7)**
Lys	60 (7)	97 (13)**	99 (15)**
Hist	30 (4)	37 (4)*	44 (7)**
Arg	71 (9)	92 (13)*	87 (12)*

Results are for 11 acute hypoxia experiments. † 15 min before hypoxia. Compared with prehypoxia (Wilcoxon's test): * $P < 0.05$; ** $P < 0.01$; *αAIB* α-aminoisobutyric acid.

From the 6 observations made in 4 fetus/ewe pairs, basal (prehypoxia) fetal plasma amino acid concentrations were generally higher than in ewes, with statistically significant increases in ten (Table 4). The largest difference was for serine which was 7.7 times higher in the fetuses ($P < 0.05$). Isoleucine, glutamic acid, taurine and lysine were lower in fetuses ($P < 0.05$). Hypoxia had no effect on plasma amino acids of these ewes whereas, despite the small number of samples, their fetuses had significant increases above basal ($P < 0.05$) in alanine, valine, leucine, isoleucine, phenylalanine, lysine, ornithine, methionine and histidine.

Discussion

For interpretation of clinical data, it is important to know how hypoxia and peripheral circulatory responses affect the plasma amino acid profile. In response to moderately severe hypoxia we found significant increases in 14 amino acids of fetal sheep, with the largest percentage rises in alanine, valine, leucine and isoleucine, phenylalanine, ornithine and lysine. Maternal plasma amino acids were not altered, indicating that the fetal disturbance reflected changes in fetal and not maternal amino acid turnover. Changes in the fetal circulation probably account for the abnormalities observed.

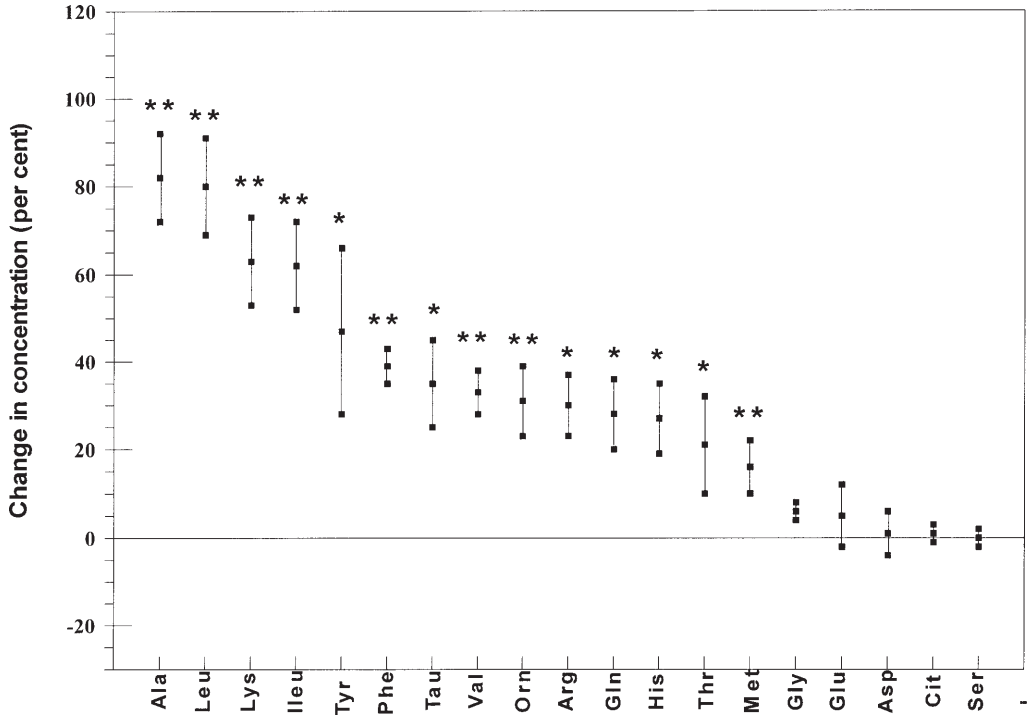

Fig. 1. Per cent change in fetal plasma amino acid concentration (mean ± SEM) during moderate hypoxia:pre-hypoxia compared with 45 min of hypoxia

Acute reduction of oxygen delivery to the fetus by around 50% leads to a major redistribution of blood flow, with centralisation in favour of the brain, heart and adrenal glands. The placental and umbilical blood flow and fetal cardiac output are maintained but peripheral blood flow falls by around 30% to 40%, with considerable diversion of blood away from fetal skeletal muscle (Boyle et al., 1990; Jensen and Berger, 1991; Itskovitz et al., 1991; Iwamoto, 1993) mediated in part by chemoreflex mechanisms (Giussani et al., 1993). Delivery of oxygenated blood to the liver also decreases substantially. Normally, about half of the (oxygenated) umbilical venous blood perfuses the liver and half bypasses it via the ductus venosus. With hypoxia, the proportion flowing through the liver falls to around a quarter (Iwamoto, 1993). Renal blood flow falls by approximately 20%, but the glomerular filtration rate is maintained (Jensen and Berger, 1991; Iwamoto, 1993; Green et al., 1997). Femoral arterial blood flow, monitored in 9 experiments in this study, decreased dramatically during hypoxia to 28% of basal control values, demonstrating a significant redistribution of blood flow. A rebound increase in flow (to 151% of basal) occurred when oxygen was restored. We have previously proposed that reflex peripheral vasoconstriction, particularly in skeletal muscle, caused significant changes in organic acid metabolism which was not due to structural organ damage (Walker et al., 1996). Peripheral vasoconstriction

Table 4. Plasma amino acids of 4 fetus/ewe pairs collected 15 min before hypoxia (results of 6 experiments)

	Plasma amino acid concentration (μmol/L)	
	Ewe mean (SEM)	Fetus mean (SEM)
Tau	88 (15)	57 (16)†
Asp	32 (5)	96 (11)*
Thre	89 (12)	183 (20)*
Ser	136 (20)	1047 (217)*
Glu	150 (11)	113 (14)†
Glmn	358 (45)	600 (26)*
Gly	465 (58)	964 (67)*
Ala	127 (15)	198 (8)*
Cit	135 (13)	117 (8)
αAIB	9 (4)	36 (11)*
Val	179 (27)	286 (32)*
Meth	23 (3)	28 (4)
Isoleu	119 (9)	84 (6)†
Leu	130 (17)	104 (10)
Tyr	43 (9)	65 (8)*
Phe	60 (7)	92 (4)*
Orn	64 (9)	59 (5)
Lys	120 (11)	56 (7)†
Hist	37 (4)	28 (2)
Arg	126 (23)	77 (9)

Significantly different (Wilcoxon's test): *$P < 0.05$; fetal concentrations *higher* than ewes'; †$P < 0.05$; fetal concentrations *lower* than ewes'.

with resulting changes in tissue metabolism would explain the significant increase in plasma lactate and associated fall in arterial pH which we observed in the fetuses. The fact that these were not apparent in ewes monitored at the same time indicates that the circulatory changes are characteristic of the fetus.

The significant rise in alanine ($P < 0.001$) is probably explained by increased release from hypoxic muscle and by decreased hepatic clearance. Skeletal muscle synthesises alanine very actively by transamination of pyruvic acid (Goldberg and Chang, 1978), probably with amino groups from branched-chain amino acids transferred via 2-oxoglutarate (Harper et al., 1984). Alanine is released from the hind limbs of normoxic fetal sheep (Leichty et al., 1987) and we propose that efflux from muscle would be higher when perfusion is reduced because of accelerated pyruvate production by glycolysis. Since umbilical blood flow is maintained (Itskovitz et al., 1991; Iwamoto, 1993), decreased placental clearance probably made a minor contribution, at most.

Skeletal muscle has high branched-chain amino acid transaminase activity and significant capacity for branched 2-oxo acid oxidation (Spydevold, 1979; Hutson and Harper, 1981; Harper et al., 1984). Because of its bulk, muscle has

a major impact on branched-chain amino acid turnover. Fetal sheep hind limb takes up all three branched-chain amino acids and normally releases less than 10% as 2-oxo acids. Their oxidation accounts for around 20% of total hind limb oxygen uptake and more during maternal fasting (Leichty et al., 1987; van Veen et al., 1987). A high NADH:NAD ratio in hypoxia inhibits branched-chain 2-oxo acid dehydrogenase. Valine catabolism is also inhibited at other sites in the degradative pathway (Walker et al., 1996). Decreased uptake and catabolism of branched-chain amino acids by ischaemic muscle would explain the raised plasma concentrations.

The increases in phenylalanine, tyrosine, lysine and ornithine probably reflect reduced hepatic blood flow. Oxygen uptake by fetal liver decreases linearly as the oxygen supply falls (Boyle et al., 1990). An increased NADH:NAD ratio would inhibit saccharopine dehydrogenase and hence lysine oxidation. Ornithine might be particularly sensitive to decreased hepatic blood flow, since ornithine aminotransferase is expressed in hepatocytes surrounding the central veins of hepatic lobules (Valle and Simmell, 1995). These are the liver cells most vulnerable to hypoxia (Sherlock and Dooley, 1997).

Are these observations in sheep relevant to man?

Plasma amino acid profiles are similar in the human and ovine fetus, except for a disproportionately higher level of serine in sheep (Yudilevich and Sweiry, 1985; Jones and Rolph, 1985). Changes in heart rate and rhythm in distressed human fetuses indicate that chemoreceptor-driven reflexes occur, and Doppler ultrasound studies of the middle cerebral arteries and aorta show clear evidence of "brain sparing" during hypoxia and hence redistribution of blood flow (Alkalin-Sel et al., 1994; Luzi et al., 1996). Increased plasma lactate at birth may be evidence for previous muscular vasoconstriction (Nelson, 1976). Postnatally any severe disorder which leads to peripheral circulatory shut-down, notably severe dehydration or acute cardiac failure, will lead to skeletal muscle hypoxia, possibly with liver hypoxia. We can predict from our present observations, that amino acid disturbances similar to those of hypoxic fetal sheep might contribute to an abnormal plasma profile. It is widely accepted that plasma alanine is raised, often, in conditions causing lactic acidaemia. One published study of plasma samples collected pre-terminally from 7 acutely ill babies and 4 at autopsy reported significantly raised alanine and glutamine with variable increases in other amino acids including lysine, but not the branched-chain amino acids (Briddon and Oberholzer, 1986). Other published data are lacking.

Our findings extend our knowledge of the fetal response to hypoxic stress and are therefore important to those exploring the concept of the fetal origins of adult disease (Barker, 1995). They demonstrate the importance of skeletal muscle in branched-chain amino acid metabolism and should help in evaluating abnormal amino acid profiles from sick infants and children.

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