Placenta 30 (2009) 974–980

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Changes in the Metabolic Footprint of Placental Explant-Conditioned Culture Medium Identifies Metabolic Disturbances Related to Hypoxia and Pre-Eclampsia

W.B. Dunn [a,1], M. Brown [b,1], S.A. Worton [c,1], I.P. Crocker [c], D. Broadhurst [d], R. Horgan [c,d], L.C. Kenny [d], P.N. Baker [c], D.B. Kell [a,b], A.E.P. Heazell [c,*,1]

[a] Manchester Centre for Integrative Systems Biology, School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, M1 7DN, UK
[b] Bioanalytical Sciences Group, School of Chemistry, Manchester Interdisciplinary Biocentre, University of Manchester, M1 7DN, UK
[c] Maternal and Fetal Health Research Group, Manchester Academic Health Science Centre, The University of Manchester, St Mary's Hospital, Manchester, M13 9WL, UK
[d] Department of Obstetrics and Gynaecology, University College Cork, Cork University Maternity Hospital, Wilton, Cork, Ireland

ARTICLE INFO

Article history:
Accepted 21 August 2009

Keywords:
Metabolomics
Metabolic footprint
Placental villous explants
Oxygen tension
Hypoxia
Pre-eclampsia

ABSTRACT

Pre-eclampsia (PE) is a multi-system disorder thought to be mediated by circulating factors released from damaged placental villous trophoblast. There is extensive evidence of changes in the villous tissue in PE, some of which may be replicated by culturing villous tissue in hypoxic conditions. Metabolic footprinting offers a hypothesis-generating strategy to investigate factors released from this tissue *in vitro*. This study investigated differences in the factors released from villous trophoblast from uncomplicated pregnancies ($n = 6$) and those with PE ($n = 6$). In both cases, explanted placental villous fragments were cultured for 96 h in 1% O_2 (hypoxia) or 6% O_2 (placental normoxia). Metabolites consumed from and released into serum-conditioned culture medium were analysed by Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC–MS). The relative concentration of 154 features of the metabolic footprint were observed to change in culture medium from uncomplicated pregnancies cultured in normoxic and hypoxic conditions ($p < 0.00005$). 21 and 80 features were also different in culture medium from PE versus uncomplicated pregnancies cultured in hypoxic and normoxic conditions, respectively ($p < 0.00005$). When comparing all 4 groups, 47 metabolic features showed a similar relative concentration in PE-derived media cultured in normoxic conditions to conditioned media from normal villous tissue cultured in hypoxic conditions. These data suggest that hypoxia may have a role in the placental pathogenesis of PE. Three areas of metabolism were highlighted for systems biology investigation; glutamate and glutamine, tryptophan metabolism and leukotriene or prostaglandin metabolism.

1. Introduction

Pre-eclampsia (PE) is a multi-system disorder of human pregnancy responsible for 60–70,000 maternal deaths worldwide per annum and 16% of intra-uterine fetal deaths in the developed world [1]. In addition, PE increases the probability of cardiovascular disease and type 2 diabetes in adult life [2]. The clinical syndrome is temporally characterised by inadequate placental–maternal adaptation followed by the release of endothelial activating factors and subsequent systemic maternal endothelial dysfunction [3]. These factors are thought to be present in the maternal circulation as plasma from women with PE can evoke endothelial dysfunction *in vitro* [4]. Despite significant research efforts the identity of these proposed pathogenic factor(s) remains elusive. Understanding these factor(s) may provide insights into the pathophysiology of PE and potentially identify therapeutic targets. Although the majority of studies have focused on changes in vasoactive proteins and peptides, changes in the metabolic composition of maternal plasma in PE have been observed [5]. Nevertheless, interpretations of metabolic changes in plasma and urine at the time of disease are complicated due to multi-organ dysfunction and difficulty in discriminating causative factors from those altered by disease processes.

The placenta is the proposed source of these causative factors, as its presence but not that of the fetus, is essential for the development of PE and prevents its resolution [6,7]. Placental changes, evident in the 1st trimester, pre-date clinical symptoms [8]. Such changes include inadequate trophoblast remodelling of maternal uterine spiral arteries which limits placental blood flow [9]. The

* Corresponding author. Maternal and Fetal Health Research Group, St Mary's Hospital, Hathersage Road, Manchester, M13 0JH, UK. Tel.: +44 161 276 5460; fax: +44 161 276 6134.
E-mail address: alexander.heazell@manchester.ac.uk (A.E.P. Heazell).
[1] These authors contributed equally to this work.

0143-4004/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2009.08.008

villous trophoblast, which has an extensive interface with the maternal circulation, exhibits several changes in PE including: increased apoptosis and degeneration of the syncytiotrophoblast with increased shedding of trophoblast-derived material into the maternal circulation [10–12]. It is hypothesised that this placental phenotype results from reduced utero-placental blood flow causing hypoxic stress within the placenta, which in turn leads to the release of pathological factors into the maternal circulation. This is supported by culture of villous tissue in hypoxic conditions (<3% O_2), which produces effects that is parallel to those seen in PE [10,13], including: increased sflt-1 levels, increased apoptosis and the release of unidentified factors into culture medium which recreate endothelial dysfunction *in vitro* [14–16]. Although plausible, the heterogeneity of clinical PE makes it unlikely that this is the only mechanism involved; as *in vitro* models of hypoxia are unable to fully recreate all placental villous changes observed in PE and do not account for the increased apoptotic susceptibility of villous tissue from PE pregnancies following exposure to hypoxia [17]. This susceptibility may reflect fundamental differences in villous tissue in PE. Therefore, factors released by villous tissue in response to hypoxia and in PE merit further investigation, regarding their potential role in mediating the maternal features of this syndrome.

We have recently developed an *in vitro* method for culturing villous tissue coupled with a metabolomic strategy for detecting and identifying metabolites released or consumed by the tissue from uncomplicated pregnancies after a hypoxic insult [18]. Metabolomics describes the study of low molecular weight chemicals (metabolites) present in, introduced to or secreted from a biological system [19]. Assessment of the intra-cellular metabolome requires technically demanding processes including: metabolic quenching, cell lysis and metabolite extraction. An alternative method is to study the extra-cellular metabolome which is easier to sample and analyse, and allows high-throughput investigations. This approach uses tissue or cell culture in a metabolite-complex medium, allowing the intra-cellular metabolism to be investigated by analysis of the extra-cellular metabolome – an approach termed 'metabolic footprinting' [20]. With regard to PE, this allows an artificial placental culture system to be investigated in which the culture medium is analogous to maternal blood and metabolites consumed by or released from villous tissue can be studied. This strategy assumes that specific areas of metabolic interest are unknown and therefore data is acquired on a large number of metabolites and interrogated to ascertain specific metabolic differences subsequent to data acquisition, an inductive or hypothesis-generating strategy [21]. Inductive metabolomic strategies have been successfully employed to identify metabolic differences in maternal plasma obtained from uncomplicated and PE pregnancies and conditioned culture medium from villous trophoblast cultured in different oxygen tensions [5,18,22].

In the current investigation we aimed to further develop placental metabolic footprinting to investigate changes in cultured villous tissue from PE and normal pregnancies cultured under normoxic and hypoxic conditions. The primary objective was to investigate the differences in the metabolic footprint associated with PE and to determine whether hypoxic conditions have a role to play in the metabolic processes of placental tissue in PE. The secondary objective of this study was to identify metabolites or metabolic pathways which merit further investigation – a hypothesis-generating approach.

2. Methods and materials

Unless stated all chemicals were obtained from Sigma–Aldrich Chemical Company (Poole, UK).

2.1. Culture of placental villous explants

Placentas were obtained with written maternal consent following approval from the local research ethics committee. Placentas were collected from women with uncomplicated, term pregnancies resulting in delivery of a healthy singleton fetus ($n = 6$) delivered by either Caesarean section (CS; $n = 3$) or normal vaginal delivery (NVD; $n = 3$) and from women with clinically defined PE ($n = 6$) delivered by either Caesarean section (CS; $n = 2$) or normal vaginal delivery (NVD; $n = 4$). PE was defined as new-onset hypertension greater than 140/90 mmHg with proteinuria greater than 0.3 g/24 h after 20 weeks gestation [23]. Pregnancies complicated by any other maternal or fetal factor, including pre-gestational hypertensive disorders and intra-uterine growth restriction were excluded. There was no significant difference between the gestational age at delivery between normal and PE pregnancies.

Placentas, received within 20 min of delivery, were sampled at three stratified areas; centre, edge, and mid-way between these points. Following removal of chorionic and decidual surfaces, portions of villous tissue (~10 mg) – termed explants – were cut from the trophoblast layer. After preparation, explants were washed briefly in phosphate buffered saline. Three explants derived from a single site were cultured separately on Netwell inserts as previously described in Ref. [15] in supplemented CMRL-1066 medium (a serum-based media) for 48 h at 37 °C in an atmospheric oxygen (O_2) tension of 1% or 6% O_2 generated by hypoxic chamber (Coy Laboratory products, Grass-Lake, MI, USA) and tri-gas incubator (Sanyo Biomedical, Loughborough, UK), respectively. For the purposes of these experiments, 6% O_2 was regarded as normoxic and 1% O_2 as hypoxic conditions for the term placenta [24]. After 48 h, culture medium was replaced with fresh equilibrated medium, and after a further 48 h, conditioned culture medium was collected and immediately frozen (−80 °C). A total of 72 samples were processed and stored.

2.2. Ultra Performance Liquid Chromatography–Mass Spectrometry (UPLC–MS) analysis

Samples of conditioned culture medium were prepared by thawing on ice, 100 μl of metabolic footprint sample was lyophilised and reconstituted in 200 μl water prior to analysis. Samples were analysed within 48 h of reconstitution in a random order using an Ultra Performance Liquid Chromatography (UPLC Acquity, Elstree, UK) coupled on-line to an electrospray LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher Scientific, Bremen, Germany) as previously described in Ref. [25]. 10 μL aliquots of each sample were introduced onto an Acquity UPLC Ethylene Bridged Hybrid (BEH) 1.7 μm-C_{18} column and eluted at a flowrate of 0.36 (negative ion mode, ES−) or 0.4 mL min^{-1} (positive ion mode, ES+) with a curvilinear gradient using a binary solvent system (water + 0.1% formic acid (A) and methanol + 0.1% formic acid (B)). The LTQ-Orbitrap hybrid mass spectrometer was operated in positive (ES+) and negative (ES−) ion modes with operating parameters tuned for maximum sensitivity for MRFA (Sigma–Aldrich, UK) at mass 514.28 in ES− and 524.14 in ES+. The Orbitrap mass analyser was mass calibrated as defined by the manufacturers. Samples and UPLC column were maintained at temperatures of 4 °C and 50 °C, respectively. A quality control (QC) sample, composed of equal aliquots of all experimental samples combined, was analysed intermittently throughout the analytical run to determine technical precision, as described previously in Ref. [25].

All UPLC–MS raw data (in. raw file format) were converted to netCDF file format with the FileConverter program available in XCalibur (ThermoFisher Scientific, Bremen, Germany) and deconvolved as previously described in Ref. [26]. This produced a list of features with associated retention time, accurate mass and chromatographic peak area. The data is not quantitative as calibration curves for each detected feature have not been experimentally acquired. The data is reported as responses, calculated as the chromatographic peak area. The relative difference between sample classes is reported as an indication of the level of difference between the median values of two sample classes.

2.3. Metabolite identification

Processed data is described by individual metabolite features (chromatographic peaks described by an accurate mass, retention time and peak area). Multiple features can represent a single metabolite. Therefore, raw data are represented by metabolite features and not metabolites. Metabolite features described in the raw dataset were putatively or definitively identified as metabolites in the processed dataset. Putative identification involved the matching of the measured accurate mass to accurate mass(es) present in the Manchester Metabolomics Database (MMD) and as previously described in Ref. [26]. Definitive identification involved the matching of accurate mass and retention time of the metabolite to that of authentic chemical standards analysed under identical conditions. The reporting of multiple metabolites for a single feature is a result of several metabolites having the same accurate mass (isomers) which have not been analysed as authentic standards and have identical retention times. Also a metabolite can be detected as different ion types (for example, a protonated species in positive ion mode and a deprotonated ion species in negative ion mode). Only chromatographic peaks assigned a chemical identity as a metabolite are reported, chromatographic peaks not assigned a chemical identity were not reported.

2.4. Statistical analysis

Univariate analysis was performed using the Mann–Whitney *U* test and the Friedman test to investigate the concentration of individual metabolites in each sample group. *P*-values of <0.00005 and <0.05 were used for the Mann–Whitney *U* test and Friedman test, respectively, to define statistical significance in a single comparison.

3. Results

Conditioned culture media from pre-eclamptic explants exhibits differences in metabolic footprint compared to conditioned media from uncomplicated pregnancies.

Previous research, using gas chromatography–mass spectrometry, has shown that differences in the metabolic footprints were observed when villous tissue from uncomplicated pregnancies were cultured at oxygen tensions of 1% O_2 (hypoxia) and 6% O_2 (normoxia) [18]. The identical research was performed applying UPLC–MS as the analytical platform. The results indicate a wider range of differences detected in the metabolic footprint, 154 metabolite features showed a statistically significant difference between 6% and 1% O_2 ($p < 0.00005$). These data are included as supplementary information (Supplementary Table 1). No discernable differences existed between samples taken from the edge, centre or midpoint within each placenta (data not shown). Therefore, data for all three sampling positions were included as replicates for further data analysis. The results demonstrate how a single metabolite feature could be multiple metabolites, for example, hydroxyadipic acid and hydroxymethylglutaric acid. In these cases further experimental work will be required to provide a unique and definitive chemical identification.

To address the primary objective, tissue-conditioned culture medium (metabolic footprint) obtained from uncomplicated and PE pregnancies under hypoxic and normoxic culture conditions were compared. Samples of unconditioned culture medium ($n = 3$ for each placenta sampled) were first analysed to determine whether the concentration of metabolic factors were altered by culture conditions. Univariate analysis was then used to compare (a) villous tissue from uncomplicated and PE pregnancies cultured in 6% O_2 and (b) villous tissue from uncomplicated and PE pregnancies cultured in 1% O_2. The results describe a range of metabolite features differing in response between subject groups in response to the same O_2 tension. These metabolites are shown in Table 1 (6% O_2) and Table 2 (1% O_2). In total, 80 features (2.5% of all detected) showed a statistical difference in normoxia (17 of which were chemically identified) and 21 (0.65% of all features detected) in hypoxia (5 of which were chemically identified). The results again highlighted how a single metabolite feature could represent multiple metabolites, e.g. multiple prostaglandins or leukotrienes for one feature.

Metabolites in conditioned media from pre-eclamptic pregnancies cultured under normoxic conditions showed similarities to that of uncomplicated pregnancies cultured under hypoxic conditions.

Further analysis of the metabolic footprints in conditioned media from uncomplicated and PE pregnancies revealed 51 metabolite features which exhibited a different pattern in their response to reduced O_2 tension. 47 of these (93%) showed a similar response in media from PE tissue cultured at 6% O_2 to media conditioned by villous tissue cultured under 1% oxygen (uncomplicated or PE). These metabolites show no statistically significant difference ($p < 0.00005$) when comparing media from PE tissue cultures at 6% O_2 and media from uncomplicated tissue cultured at 1% O_2. The metabolites exhibiting this pattern which have been chemically identified are shown in Table 3. In contrast to the differences seen between normal and PE conditioned media at 6% O_2 the relative differences for the reported metabolites in the metabolic footprint of PE pregnancy tissue cultured in 6% O_2 and the metabolic footprint of uncomplicated pregnancy tissue cultured in 1% O_2 were observed in the range 0.91–1.13 and are close to 1.0. Two examples, glutamate and oxypurinol, are shown in Fig. 1. Glutamate shows a lower concentration in PE and in 1% O_2, conversely oxypurinol shows higher concentrations in PE and in 1% O_2. Two examples, glutamate and oxypurinol, are shown in Fig. 1. Glutamate shows a lower concentration in PE and in 1% O_2, conversely oxypurinol shows higher concentrations in PE and in 1% O_2. The presence of glutamate in these samples was confirmed by

Table 1
The range of chemically identified statistically different metabolites ($p < 0.00005$) observed in the metabolic footprint of villous tissues from uncomplicated and PE pregnancies cultured in normoxia (6% O_2). A single metabolite feature can represent more than one metabolite and in these cases all metabolites are reported. Metabolites matched to the accurate mass (±5 ppm) and retention times (±15 s) of an authentic standard are described as definitive identifications. Metabolites matched to the accurate mass (±5 ppm) only are described as putative identifications. The relative difference is calculated as the median peak area response in the metabolic footprint of PE pregnancy tissue/median peak area in the metabolic footprint of uncomplicated pregnancy tissue. A value greater than one describes a higher concentration in the metabolic footprint of PE pregnancy tissue compared to the metabolic footprint of uncomplicated pregnancy tissue.

Identification type	Metabolite	Relative difference [PE 6%/Normal 6%]
Definitive	Glutamine	0.42
Definitive	Cystamine	0.52
Definitive	Hydroxyphenyllactate	0.59
Definitive	Kynurenine	0.63
Definitive	Hydroxypalmitate	0.66
Definitive	Hydroxystearate	0.72
Definitive	Glutamate	0.76
Putative	Methyl oxalate or methylmalonate or succinate	0.42
Putative	Arginine phosphate	0.47
Putative	Prosataglandin or leukotriene	0.55
Putative	3,4-Dihydroxy-1-methylquinolin-2(1 h)-one or 5,6-dihydroxy-3-methyl-2-oxo-1, 2-dihydroquinoline or 5-hydroxyindoleacetate or indole-3-glycolate or hydroxydopamine or adrenaline or noradrenaline or pyridoxine	0.61
Putative	5-Amino-3-methyl-pyrrolidine-2-carboxylic acid or formylsalicylic acid	0.65
Putative	Methylaspartate or 2-oxo-4-hydroxy-5-aminovalerate or glutamate or acetylserine	0.73
Putative	1-Phosphatidyl-myo-inositol	0.77
Putative	Glucosamine 6-sulfate	0.81
Putative	Glycerophosphocholine	0.81
Putative	N1,N12-Diacetylspermine	2.10

Table 2
The range of chemically identified statistically different metabolites ($p < 0.00005$) observed in the metabolic footprint of villous tissues from uncomplicated and PE pregnancies cultured in hypoxia (1% O_2). Metabolites matched to the accurate mass (±5 ppm) and retention times (±15 s) of an authentic standard are described as definitive identifications. Metabolites matched to the accurate mass (±5 ppm) only are described as putative identifications. The fold difference is calculated as the median peak area response in the metabolic footprint of PE pregnancy tissue/median peak area in the metabolic footprint of uncomplicated pregnancy tissue. A value greater than one describes a higher concentration in the metabolic footprint of PE pregnancy tissue compared to the metabolic footprint of uncomplicated pregnancy tissue.

Identification type	Metabolite	Relative difference [PE 1%/normal 1%]
Definitive	Progesterone	1.91
Definitive	Glycerol	1.92
Putative	Valinol or choline	1.40
Putative	Diglyceride	1.51

gas chromatography–mass spectrometry analysis to eliminate other possible identities. In contrast, only 4 metabolites (7%) detected in media conditioned by PE villous tissue showed a normoxic phenotype when cultured in 1% O_2.

4. Discussion

Metabolomics has proven to be an appropriate tool to study differences in the metabolome of maternal plasma or urine associated with PE [5,27]. Although previous studies have harnessed the potential of metabolomic techniques to detect hypoxic phenotypes in placental tissue cultured under hypoxic conditions [18] and in high-altitude pregnancies [28], the novelty of these techniques necessitates on-going investigations with regard to placental function. Using media conditioned by explanted villous tissue from uncomplicated pregnancies and those complicated by PE this study confirmed differences in the metabolic footprint of media conditioned by hypoxic conditions. In addition, media conditioned by PE villous tissue had a similar metabolic footprint at normoxic conditions to media conditioned by normal villous tissue cultured under hypoxic conditions. It should be noted that cultured tissues were from late-onset PE and tissue metabolism may change between early and late stages of PE. Further research is required to ascertain whether the metabolic differences discussed are observed in early stages of PE or even before disease is evident.

Using other experimental techniques and *in vitro* culture models, placental tissues cultured under hypoxic conditions have been shown to have similarities to tissues from pregnancies complicated with PE [11,14,15]. Making use of novel metabolomic strategies to broaden these studies, two patterns emerge. Firstly, a greater proportion of metabolic differences are evident between the metabolic footprints of normal and PE after culture under normoxic conditions as compared to hypoxic conditions (6% versus 1%). Secondly, 47 metabolite features detected in the media of villous tissues from placentas of PE pregnancies, cultured at 1% O_2 and 6% O_2 (placental hypoxic and normoxic) showed striking similarities to normal placental tissues exposed to hypoxia, i.e. suggestive of a "hypoxic" profile in PE. These metabolites showed the same response in conditioned media from placental explants from PE cultured under normoxic and hypoxic conditions and

Table 3
Identified metabolites showing the same response in the metabolic footprints of PE tissues cultured under normoxic and hypoxic conditions and tissues from uncomplicated pregnancies cultured under hypoxic conditions. These metabolites show no statistically significant difference ($p < 0.00005$) when comparing media from PE tissue cultures at 6% O_2 and media from uncomplicated tissue cultured at 1% O_2. A single metabolite feature can represent more than one metabolite and in these cases all metabolites are named. Metabolites matched to the accurate mass (±5 ppm) and retention times (±15 s) of an authentic standard are described as definitive identifications. Metabolites matched to the accurate mass (± 5 ppm) only are described as putative identifications. In the third column the relative fold difference is calculated as the median peak area response in the metabolic footprint of PE pregnancy tissue cultured in normoxia/median peak area in the metabolic footprint of uncomplicated pregnancy tissue cultured in normoxia. A value greater than one describes a higher concentration in the metabolic footprint of PE pregnancy tissue compared to the metabolic footprint of uncomplicated pregnancy tissue. The relative differences for the reported metabolites for a comparison of the metabolic footprint of PE pregnancy tissue cultured in normoxia and the metabolic footprint of uncomplicated pregnancy tissue cultured in hypoxia were observed in the range 0.91–1.13 and are close to 1.0.

Identification type	Metabolite	Relative difference [PE 6%/normal 6%]
Definitive	Glutamine	0.42
Definitive	Cystamine	0.52
Definitive	Kynurenine	0.63
Definitive	Hydroxypalmitate	0.66
Definitive	Hydroxystearate	0.72
Definitive	Histidine	0.81
Putative	Prosataglandin or leukotriene	0.55
Putative	3-(4-Hydroxyphenyl)lactate or 2',6'-dihydroxy-4'-methoxyacetophenone or 3-methoxy-4-hydroxyphenylglycolaldehyde or dihydrocaffeic acid or homovanillate	0.59
Putative	2-Methylacetoacetic acid or oxopentanoic acid or levulinic acid	0.60
Putative	Methionine phosphinate	0.63
Putative	5-Amino-3-methyl-pyrrolidine-2-carboxylic acid or formylsalicylic acid	0.66
Putative	Isopropylmalate or 2,3-dimethyl-3-hydroxyglutaric acid	0.66
Putative	2-(Sulfomethyl)thiazolidine-4-carboxylate	0.70
Putative	2-Quinolinecarboxylic acid or indole-3-glyoxal	0.70
Putative	2',3'-Cyclic UMP or 4,7-dioxosebacic acid	0.71
Putative	Glutamate	0.73
Putative	Glutamate	0.73
Putative	Methionine phosphonate or methionine	0.75
Putative	2-Phenylglycine or dopamine quinone or N-METHYL-4-aminobenzoate or N-methylanthranilate	0.76
Putative	10,11-Dihydro-12-hydroxy-leukotriene E4	1.28
Putative	Pantolactone or methyl-2-oxopentanoate or oxohexanoic acid	1.31
Putative	O-phosphoethanolamine	1.34
Putative	Diglyceride	1.37
Putative	Enkephalin L	1.43
Putative	Oxypurinol	1.45

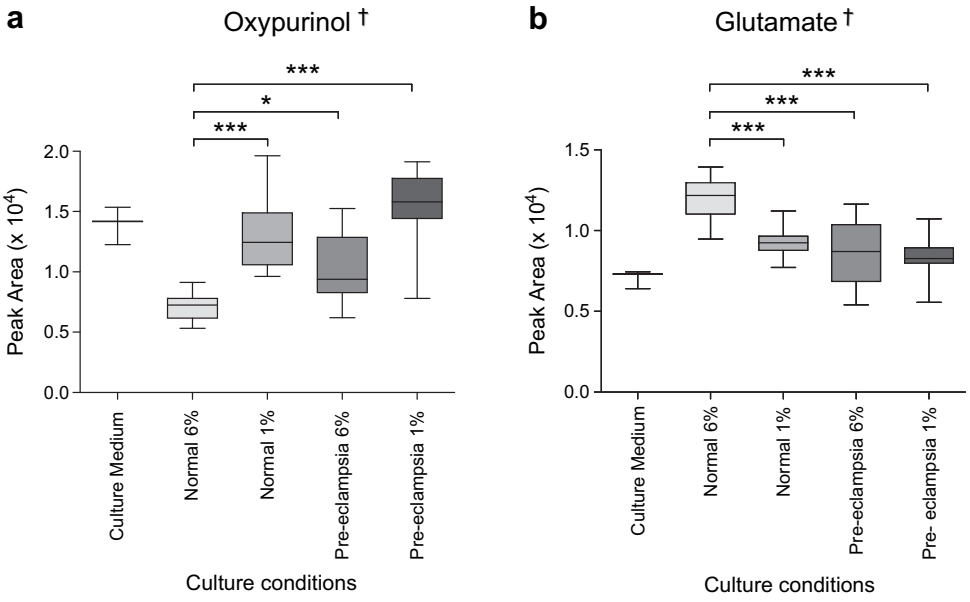

Fig. 1. Box and whisker plots showing the peak area of (a) oxypurinol and (b) glutamate for different sample classes $^{*}p < 0.05$, $^{***}p < 0.001$. †These are putative identifications only. The box and whisker plots are non-parametric descriptions of the distribution of data. The box describes the upper and lower quartiles of the data with the median described as the horizontal line in the box. The whiskers describe the highest and lowest observed values.

tissue from uncomplicated pregnancies cultured under hypoxic conditions. The response was however different for media from uncomplicated pregnancies cultured under normoxic conditions. Taken together, these findings indicate that only specific aspects of placental metabolism are altered in PE, rather than global changes affecting all metabolic pathways. Furthermore, this altered metabolism appears to be irreversible under the culture conditions used here, suggesting the explanted placental tissue from PE pregnancies already shows a hypoxic phenotype when sampled.

Metabolic features from a wide range of metabolite classes including amino acids, fatty acids and prostaglandins/leukotrienes were differentially identified between study groups and O_2 levels. The biological considerations of all these metabolites cannot be discussed in detail; but three classes, altered in responses between normoxic and hypoxic culture, and observed in the "hypoxic" profile of the villous placenta in PE, will be further considered below: (a) glutamic acid and glutamine (b) prostaglandins and leukotrienes and (c) kynurenine metabolism. Further research is being performed to increase the accuracy of metabolite identification for class (b) prostaglandins and leukotrienes. However, these structurally similar metabolites cannot be uniquely identified with the protocols employed and so both metabolite classes will be discussed. A systems biology strategy is to be utilised to investigate the importance and interactions of these metabolites within the placental proteome and transcriptome [29].

Glutamic Acid (glutamate), a non-essential amino acid, is found at lower levels in maternal plasma in uncomplicated pregnancies compared to pregnancies complicated by PE [5]. The presence of amino acid acidaemia in plasma of eclamptic and pre-eclamptic subjects has also been reported, where significant differences in concentration were observed for threonine, arginine, glycine, cystine, glutamic and aromatic amino acids [30]. In the studies described herein, glutamic acid was available in culture medium prior to experimentation and was further elevated after culture. This increase was maximal under normoxic conditions and for tissue from uncomplicated pregnancies. It therefore appears that glutamic acid was generated at a lower rate or consumed at a higher rate in hypoxic cultures and those of PE origin. In a similar manner, glutamic acid is taken up or consumed at higher rates in cardiomyocytes under hypoxic conditions [31]. Like glutamic acid, glutamine, its amide derivative, is present at decreased concentrations in maternal plasma in PE [32] and was similarly reduced in the media from hypoxic cultures and those from PE-derived placental tissues. Other studies have shown that the low glutamine concentration of PE can result in greater CAM (cell adhesion molecule) expression and enhanced trans-endothelial migration of neutrophils, potentially key features of the syndrome. Glutamine administration can reduce CAM expression and decrease IL-8 production in this context [33].

Prostaglandins are paracrine lipid-mediators which can act upon many different cell types. They have a variety of functions including dilation of vascular smooth muscle [34,35] and the regulation of inflammatory mediation [36]. Prostaglandins are biosynthesised from sequential oxidation of lipids (gamma-linolenic acid (GLA), arachidonic acid (AA) and eicosapentaenoic acid (EPA)) typically by cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2). Research has shown that the activities of COX-1 and COX-2 are decreased in placental villous tissue in PE, presumably caused by oxidative stress [37]. Our results suggest that this decrease in COX activity translates to the metabolome where lower concentrations were observed in PE-derived media and in normal media in response to hypoxia. These metabolites were not detected in the growth medium before culture, which demonstrates that prostaglandins are produced de novo by villous tissue. Prostaglandins have also been shown to influence concentration dependent vasoconstriction of placental and myometrial vessels [38], and have also been highlighted in the molecular mechanisms of ineffective implantation and early placentation [39].

The role of leukotrienes in hypoxia (and PE) appears to be linked to the transcription of nitric-oxide synthase (iNOS) [40,41]. Chemical or physical hypoxia has been shown to increase gene transcription of iNOS and subsequent NO production. This increase has also been shown to perpetuate lipid peroxidation and leukotriene B4 (LTB_4) generation. LTB_4 is suppressed in pregnancy to

inhibit the activation of neutrophils and other leukocytes. Conversely, this inhibition is not observed in PE and the failure of this mechanism may contribute to the maternal inflammatory syndrome [42].

In these culture studies, the response of kynurenine was decreased in conditioned media from PE placental tissue and in hypoxia, perhaps pointing to reduced catabolism of tryptophan in these conditions. Decreased catabolism of tryptophan has been suggested by reduced placental indoleamine 2,3-dioxygenase activity [43]. Like LTB_4, these changes may initiate a dysregulation of the inflammatory response, thus contributing to the maternal syndrome of PE [44]. Other studies have shown a decrease in 5-hydroxyindoleacetate (present in the same pathway as kynurenine) concentration in plasma and urine in PE [45]. Alternatively, a reduction in tryptophan could be due to transformation to 5-hydroxytryptamine (serotonin) which is increased in the placenta in PE pregnancies [46]. This highlights the issue of whether the changes observed in maternal plasma or urine are indicative of placental or kidney dysfunction. Notably, placental compromise has also been shown to influence kynurenine metabolism in the brain of the fetal sheep [47].

This study shows the potential application of metabolomic strategies to investigate differences associated with disease pathophysiology and identify novel substances that merit further investigation. The findings support the utility of metabolomic investigations in placental research and demonstrate the ability of hypoxic culture conditions to recreate a PE phenotype in villous explants. This investigation is restricted by small sample sizes and the potential role of individual metabolites must be interpreted with caution due to increased risks of Type 1 errors associated with multiple-hypothesis testing. We have limited this possibility with stringent deconvolution criteria and a low p-value ($p < 0.00005$) to define statistical significance. Another source of confounding may be the use of tissues obtained from Caesarean section or normal vaginal delivery. It may be expected that the two different delivery modes would alter the placental tissue metabolism caused by different stresses during delivery, including different oxygen tensions. Combining data from this and our previous study we have not been able to detect any difference between samples from normal birth and Caesarean section. The authors suggest that 96 h culture supersedes the effects of mode of delivery.

Ultimately, repetition of this study in an independently obtained sample set is required to validate observations reported as has been described previously in Ref. [18]. Other work will be performed to confirm the identity of features reported with multiple putative identifications, including tandem mass spectrometry (MS/MS) and the analysis of authentic chemicals standards to confirm that the retention time and MS/MS mass spectra of standards and metabolite are identical.

Overall, this investigation advances the emerging field of placental metabolomics by employing methods to investigate the metabolic footprint of complicated pregnancies. This form of metabolic footprinting has successfully identified differences in the profile of factors released from villous tissues from pregnancies complicated by PE; it remains to be determined whether the information derived can lead to robust hypothesis-generated discoveries. Nevertheless, the investigations described here reinforce twin pillars of existing evidence which implicate; (i) intrinsic changes in the villous tissue in this syndrome, and (ii) a role for hypoxia or similar response in the related-placental pathogenesis. This confirms our understanding of PE as a multi-factorial condition but also highlights the need for a systems-wide study of the transcriptome, proteome and metabolome and their interactions in this complex pregnancy disorder.

Acknowledgements

The authors would like to thank the midwives on Central Delivery Unit, St Mary's Hospital, Manchester for their assistance in acquiring placental tissue. This work was funded by the BBSRC, the British Heart Foundation, The Health Research Board, Ireland and Tommy's–the baby charity. We also wish to acknowledge support from the NIHR Biomedical Research Centre, Manchester. WBD and DBK would like to thank the BBSRC and EPSRC for financial support of The Manchester Centre for Integrative Systems Biology. MB would like to thank the British Heart Foundation for financial assistance. IPC is a NIHR post-doctoral fellow. LCK is a Health Research Board Ireland Clinician Scientist.

Supplementary material

Supplementary material can be found, in the online version, at doi:10.1016/j.placenta.2009.08.008

References

[1] Myers JE, Brockelsby J. The epidemiology of pre-eclampsia. In: Baker PN, Kingdom JCP, editors. Pre-eclampsia: current perspectives on management. London: Parthenon; 2004.
[2] Forest JC, Girouard J, Masse J, Moutquin JM, Kharfi A, Ness RB, et al. Early occurrence of metabolic syndrome after hypertension in pregnancy. Obstet Gynecol 2005;105(6):1373–80.
[3] Redman CW, Sargent IL. Latest advances in understanding preeclampsia. Science 2005;308(5728):1592–4.
[4] Myers J, Mires G, Macleod M, Baker P. In preeclampsia, the circulating factors capable of altering in vitro endothelial function precede clinical disease. Hypertension 2005;45(2):258–63.
[5] Kenny LC, Broadhurst D, Brown M, Dunn WB, Redman CWG, Kell DB, et al. Detection and identification of novel metabolomic biomarkers in preeclampsia. Reprod Sci 2008;15(6):591–7.
[6] Westerhout Jr FC, Morel ES, Slate WG. Observations on 138 molar pregnancies. Am J Obstet Gynecol 1969;103(1):56–9.
[7] Piering WF, Garancis JG, Becker CG, Beres JA, Lemann Jr J. Preeclampsia related to a functioning extrauterine placenta: report of a case and 25-year follow-up. Am J Kidney Dis 1993;21(3):310–3.
[8] Prefumo F, Ayling LJ, Dash PR, Cartwright JE, Thilaganathan B, Whitley GSJ. Uterine artery resistance at 10–14 weeks of gestation is correlated with trophoblast apoptosis. Ultrasound Obstet Gynaecol 2004;24:234.
[9] Meekins JW, Pijnenborg R, Hanssens M, McFadyen IR, van Asshe A. A study of placental bed spiral arteries and trophoblast invasion in normal and severe pre-eclamptic pregnancies. Br J Obstet Gynaecol 1994;101(8):669–74.
[10] Leung DN, Smith SC, To KF, Sahota DS, Baker PN. Increased placental apoptosis in pregnancies complicated by preeclampsia. Am J Obstet Gynecol 2001;184(6):1249–50.
[11] Heazell AE, Moll SJ, Jones CJ, Baker PN, Crocker IP. Formation of syncytial knots is increased by hyperoxia, hypoxia and reactive oxygen species. Placenta 2007;28(Suppl. 1):S33–40.
[12] Knight M, Redman CW, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. Br J Obstet Gynaecol 1998;105(6):632–40.
[13] Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003;111(5):649–58.
[14] Nevo O, Soleymanlou N, Wu Y, Xu J, Kingdom J, Many A, et al. Increased expression of sFlt-1 in *in vivo* and in vitro models of human placental hypoxia is mediated by HIF-1. Am J Physiol Regul Integr Comp Physiol 2006;291(4):R1085–93.
[15] Heazell AE, Lacey HA, Jones CJ, Huppertz B, Baker PN, Crocker IP. Effects of oxygen on cell turnover and expression of regulators of apoptosis in human placental trophoblast. Placenta 2008;29(2):175–86.
[16] Robinson NJ, Wareing M, Hudson NK, Blankley RT, Baker PN, Aplin JD, et al. Oxygen and the liberation of placental factors responsible for vascular compromise. Lab Invest 2008;88(3):293–305.
[17] Crocker IP, Cooper S, Ong SC, Baker PN. Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with preeclampsia and intrauterine growth restriction. Am J Pathol 2003;162(2):637–43.
[18] Heazell AEP, Brown M, Dunn WB, Worton SA, Crocker IP, Baker PN, et al. Analysis of the metabolic footprint and tissue metabolome of placental villous explants cultured at different oxygen tensions reveals novel redox biomarkers. Placenta 2008;29(8):691–8.

[19] Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB. Metabolomics by numbers: acquiring and understanding global metabolite data. Trends Biotechnol 2004;22(5):245–52.
[20] Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. Metabolic footprinting and systems biology: the medium is the message. Nat Rev Microbiol 2005;3(7):557–65.
[21] Kell DB, Oliver SG. Here is the evidence, now what is the hypothesis? The complementary roles of inductive and hypothesis-driven science in the post-genomic era. Bioessays 2004;26(1):99–105.
[22] Kenny LC, Dunn WB, Ellis DI, Myers J, Baker PN, Kell DB. Novel biomarkers for pre-eclampsia detected using metabolomics and machine learning. Metabolomics 2005;1(3):227–34.
[23] Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders of pregnancy. Am J Obstet Gynecol 1988;158(4):892–8.
[24] Miller RK, Genbacev O, Turner MA, Aplin JD, Caniggia I, Huppertz B. Human placental explants in culture: approaches and assessments. Placenta 2005;26(6):439–48.
[25] Zelena E, Dunn WB, Broadhurst D, Francis-McIntyre S, Carroll KM, Begley P, et al. Development of a robust and repeatable UPLC–MS method for the long-term metabolomic study of human serum. Anal Chem 2009;81(4):1357–64.
[26] Brown M, Dunn WB, Dobson P, Patel Y, Winder CL, Francis-McIntyre S, et-al. Mass spectrometry tools and metabolite-specific databases for molecular identification in metabolomics. The Analyst 2009;134(7):1322–32.
[27] Turner E, Brewster JA, Simpson NAB, Walker JJ, Fisher J. NMR-based metabonomics – searching for novel biomarkers of preeclampsia. Hypertens Pregnancy 2006;25(suppl 1):14.
[28] Tissot van Patot MC, Burton GJ, Cindrova-Davies T, Johns J, Jauniaux E, Serkova N. Oxidative stress and energy metabolism are similar between healthy and pre-eclamptic placentas at high altitude but not between preeclamptic placentas at low vs high altitude. Placenta 2007;28(8–9):A69.
[29] Serkova NJ, Reisdorph NA, van Patot MCT. Metabolic markers of hypoxia: systems biology application in biomedicine. Toxicol Mech Methods 2008;18(1):81–95.
[30] Li J, Sun J, Lu H. Determination of serum free amino acids and ammonia in pregnant women with severe pregnancy induced hypertension. Zhonghua Fu Chan Ke Za Zhi 1996;31(8):468–70.
[31] Dinkelborg LM, Kinne RK, Grieshaber MK. Transport and metabolism of L-glutamate during oxygenation, anoxia, and reoxygenation of rat cardiac myocytes. Am J Physiol 1996;270(5):H1825–32.
[32] Hsu CS, Chou SY, Liang SJ, Chang CY, Yeh CL, Yeh SL. Effect of physiologic levels of glutamine on ICAM-1cell adhesion molecule expression and leukocyte transmigration in endothelial cells activated stimulated by preeclamptic plasma. J Reprod Med 2006;51(3):193–8.
[33] Hsu CS, Chou SY, Liang SJ, Chang CY, Yeh CL, Yeh SL. Effect of glutamine on cell adhesion molecule expression and leukocyte transmigration in endothelial cells stimulated by preeclamptic plasma. Nutrition 2005;21(11–12):1134–40.
[34] Koller A, Kaley G. Prostaglandins mediate arteriolar dilation to increased blood flow velocity in skeletal muscle microcirculation. Circ Res 1990;67(2):529–34.
[35] Lumley P, Humphrey PP, Kennedy I, Coleman RA. Comparison of the potencies of some prostaglandins as vasodilators in three vascular beds of the anaesthetised dog. Eur J Pharmacol 1982;81(3):421–30.
[36] Lawrence T, Willoughby DA, Gilroy DW. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2002;2:787–95.
[37] Borekci B, Aksoy H, Toker A, Ozkan A. Placental tissue cyclo-oxygenase 1 and 2 in pre-eclamptic and normal pregnancy. Int J Gynaecol Obstet 2006;95(2):127–31.
[38] Friel AM, Hynes PG, Sexton DJ, Smith TJ, Morrison JJ. Expression levels of mRNA for Rho A/Rho kinase and its role in isoprostane-induced vasoconstriction of human placental and maternal vessels. Reprod Sci 2008;15(2):179–88.
[39] Norwitz ER. Defective implantation and placentation: laying the blueprint for pregnancy complications. Reprod Biomed Online 2006;13(4):591–9.
[40] Kiang JG, Tsen KT. Biology of hypoxia. Chin J Physiol 2006;49(5):223–33.
[41] Kiang JG, Krishnan S, Lu X, Li Y. Inhibition of inducible nitric-oxide synthase protects human T cells from hypoxia-induced apoptosis. Mol Pharmacol 2008;73(3):738–47.
[42] Imai T, Arai T. Quantitative changes in leukotriene B4 release in neutrophilic leukocytes activated by Ca ionophore during pregnancy – normal pregnancy and preeclampsia. Nippon Sanka Fujinka Gakkai Zasshi 1996;48(6):405–11.
[43] Sedlmayr P. Indoleamine 2,3-dioxygenase in materno–fetal interaction. Curr Drug Metab 2007;8(3):205–8.
[44] Kudo Y, Boyd CA, Sargent IL, Redman CW. Decreased tryptophan catabolism by placental indoleamine 2,3-dioxygenase in preeclampsia. Am J Obstet Gynecol 2003;188(3):719–26.
[45] Schäfer CA, du Bois A, Vach W, Prömpeler H, Bauknecht T, Breckwoldt M. Changes in serotonin metabolism in pre-eclampsia. Geburtshilfe Frauenheilkd 1996;56(8):418–22.
[46] Gujrati VR, Shanker K, Vrat, Chandravati S, Parmar SS. Novel appearance of placental nuclear monoamine oxidase: biochemical and histochemical evidence for hyperserotonomic state in preeclampsia–eclampsia. Am J Obstet Gynecol 1996;175(6):1543–50.
[47] Nicholls T, Lacey B, Nitsos I, Smythe G, Walker DW. Regional changes in kynurenic acid, quinolinic acid, and glial fibrillary acidic protein concentrations in the fetal sheep brain after experimentally induced placental insufficiency. Am J Obstet Gynecol 2001;184(2):203–8.