Thyroid hormones and their placental deiodination in normal and pre-eclamptic pregnancy

Lesia O. Kurlak[1*], Hiten D. Mistry[2*], Ellen Kaptein[3], Theo J. Visser[3] & Fiona Broughton Pipkin[1]

[1]Division of Obstetrics & Gynaecology, School of Clinical Sciences, University of Nottingham, Nottingham, NG5 1PB, UK [2] Division of Women's Health, King's College London, Women's Health Academic Centre, London, SE1 7EH [3]Department of Internal Medicine, Erasmus University Medical Center, 3015 GE Rotterdam, The Netherlands.

** Authors made equal contribution to this study.*

Corresponding author's address: Dr. Lesia O. Kurlak
Division of Obstetrics & Gynaecology,
University of Nottingham,
1st Floor, Maternity Unit,
City Hospital,
Hucknall Road,
Nottingham,
NG5 1PB, UK
Lesia.kurlak@nottingham.ac.uk
Tel: +44(0)115 82 31955
Fax: +44 (0)115 82 31908

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Abstract

Pre-eclampsia is associated with lower serum selenium concentrations and glutathione peroxidase expression/activity; total thyroid hormones are also lower. ***Objectives, study design and main outcome measures:*** We hypothesised that the placental selenoprotein deiodinase (D3) will be protected in pre-eclampsia due to the hierarchy of selenoprotein biosynthesis in selenium deficiency. Venous blood and tissue from three standardised placental sites were obtained at delivery from 27 normotensive and 23 pre-eclamptic women. mRNA expression and enzyme activity were assessed for both deiodinases (D2 and D3); protein expression/localisation was also measured for D3. FT_4, FT_3 and TSH concentrations were measured in maternal and umbilical cord blood. ***Results:*** No significant differences in D3 mRNA or protein expression between normotensive and pre-eclamptic pregnancies. There was a significant effect of sampling site on placental D3 activity only in pre-eclamptic women ($P=0.034$; highest activity nearest the cord). A strong correlation between D3 mRNA expression and enzyme activity existed only in the pre-eclamptic group; further strengthened when controlling for maternal selenium ($P<0.002$). No significant differences were observed between groups for any of the maternal thyroid hormones; umbilical TSH concentrations were significantly higher in the pre-eclamptic samples ($P<0.001$). ***Conclusions:*** D3 mRNA and protein expression appear to be independent of selenium status. Nevertheless, the positive correlation between D3 mRNA expression and activity evident only in pre-eclampsia, suggests that in normotensive controls, where selenium is higher, translation is not affected, but in pre-eclampsia, where selenium is low, enzyme regulation may be altered. The raised umbilical TSH concentrations in pre-eclampsia may be an adaptive fetal response to maximise iodide uptake.

Keywords: Placenta, deiodinases, pre-eclampsia, thyroid hormones

Introduction

The availability and integration of the trace element selenium into the selenocysteine amino acid (Sec) is crucial to the enzymatic function of deiodinases (D1, D2 and D3). The regulation of selenoprotein synthesis is highly selenium-dependent; it has been shown that a hierarchy exists for the synthesis of different selenoproteins, both via differential mRNA translation and sensitivity to nonsense-mediated decay with D3 being prioritised [1]. The placenta is a key site for the activity of many selenoproteins such as the antioxidant glutathione peroxidase (GPx), iodothyronine deiodinase, and redox signalling thioredoxin reductase families [2]. Many of these roles appear to be particularly relevant to the aetiology of the pregnancy-specific condition of pre-eclampsia, a hypertensive disorder of pregnancy that occurs in ~3% of all pregnancies (*de novo* proteinuric hypertension), a leading cause of maternal and perinatal mortality and morbidity worldwide [3]. Placental and maternal systemic oxidative stress are components of the syndrome [4] and contribute to a generalised maternal systemic inflammatory activation [5]. Placental ischemia-reperfusion injury has been implicated in excessive production of reactive oxygen species, which could cause release of placental factors that mediate the inflammatory responses [6]. We have recently shown increased maternal and fetal plasma thiobarbituric acid reactive substances (TBARS) concentrations which were measured as a global marker of oxidative stress in pre-eclampsia [4].

There are three iodothyronine deiodinases, which all utilise Sec at their active site. Deiodinase types 1 and 2 (D1 and D2) primarily catalyse the removal of an iodine from the outer (phenolic) ring and in so doing convert inactive T_4 to T_3. Deiodinase type 3 (D3) catalyses the deiodination of the inner (tyrosyl) ring of both T_4 and T_3 to produce the inactive products

reverse T_3 (rT_3) and 3, 3'-diiodothyronine (T_2), respectively [7, 8]. D2 and D3 mRNA and activity have both been identified in homogenates of human placenta from near the cord insertion site [9-12]; their activity decreases with gestational age from the end of the first trimester [10, 11]. D2 is an integral membrane protein found mainly in the endoplasmic reticulum [13], while D3 is localised in the plasma membrane of the intra-placental cells; the highest levels of D3 are found in the placenta [12]. In the human feto-placental unit, D3 metabolizes T_4 to rT_3 throughout pregnancy [14]; only later in pregnancy there is an increase in T_4 to T3 conversion by D1 and D2. Fetal thyroxine-binding globulin (TBG) concentrations rise to non-pregnant levels by the late 3rd trimester, although remaining lower than maternal[15]; the fetal T_4:TBG ratio is, however, higher at term.

Placental D3 enzyme activity is 100-400 fold greater than D2 activity and the D3/D2 mRNA ratio varies from 0.5-50 [10]. Placental D2 mRNA concentrations correlate with neither protein nor activity rates [10]. Placental D3 activity is unaffected by plasma T_4 concentrations [9, 16] and is controlled by post-transcriptional and post-translational regulation [17] such as the TGF-β via Smad-dependent pathway [18].

Total T_3 and T_4, as well as TBG concentrations in women with pre-eclampsia have been reported to be lower compared to normotensive pregnant women but TSH concentrations are higher [19-21]; these changes have also been observed in fetal samples from pre-eclamptic pregnancies [22]. We have also shown maternal and umbilical venous serum selenium concentrations to be decreased in pregnancy and to be further reduced in pre-eclamptic pregnancy [4]. A strong positive relationship exists between GPx activity and serum selenium concentrations in both maternal plasma and placental tissue and we have reported significant

reductions in maternal and fetal GPx protein expression and activity in both plasma and placental tissue [4, 23]. The hierarchal control of selenoproteins appears to exist in selenium deficient conditions and ranks deiodinases higher than GPxs [24]. Systematic investigation of the placental deiodinases in relation to pre-eclampsia appears not to have been undertaken.

We hypothesised that D3 would be preserved in placentae from pre-eclamptic women despite their lower serum selenium. We also hypothesised that decreased selenium would be associated with increased TSH due to the role of deiodinases in extrathyroidal production of T_3, to maintain FT_3 and FT_4 concentrations.

Methods

Subjects: The investigations were approved by the Nottingham Hospital Ethics Committee; written, informed consent was obtained from each participant. Pre-eclampsia was defined as a systolic blood pressure of 140 mm Hg or more and diastolic pressure (Korotkoff V) of 90 mm Hg or more on 2 occasions after 20 weeks gestation in a previously normotensive woman together with proteinuria ≥300 mg/L, ≥500 mg/day or ≥2+ on dipstick analysis of midstream urine (MSU) if 24-hour collection result was not available [25]. The study population consisted of White European women who had either a normotensive (n=27) or pre-eclamptic (n=23) pregnancy (Table 1) [4]. Umbilical venous blood samples were obtained from babies from 24 of the normotensive and 14 pre-eclamptic women. Medical and obstetric histories, including delivery data, were obtained for each woman. The birthweight centile for each baby was computed, correcting for gestation age, sex, maternal parity and body mass index (BMI) [26].

Sample collection: Venous blood samples were taken from mothers before delivery; where possible, umbilical venous samples were also taken, immediately after placental delivery. Samples were taken into chilled tubes with no anticoagulant and the serum fraction stored at –80°C until required. Two full depth placental tissue samples were collected from three standardised locations between the cord insertion and placental periphery (1 cm from the cord insertion (Near), 1 cm from the periphery (Outer), and midway between the two (Middle)), avoiding placental infarcts. The placental samples were taken within 10 minutes of delivery, membranes removed and the tissue washed in ice cold 1x PBS to remove maternal blood contamination. One set of samples was snap frozen in liquid nitrogen and stored at -80 ^{o}C for measurement of deiodinase activity and RNA assessment; the other was formalin fixed and wax-embedded for immunohistochemical analysis.

Quantitative real-time PCR: Total RNA was extracted from a known amount of placental tissue (100 mg) using QIAzol lysis reagent (Qiagen, Crawley, UK). RNA concentration and quality were verified spectrophotometrically, using the Nanodrop ND-1000 (Nanodrop Technologies, Labtech, Ringmer, UK); all samples had an A_{260}/A_{280} ratio greater than 1.96 and were stored at −80 °C. RNA (1 µg) was then reverse transcribed using the QuantiTect Reverse Transcription Kit containing a mix of random primers and Oligo dT (Qiagen, Crawley, UK) in a Primus 96 advanced gradient thermocycler (Peqlab Ltd, Fareham, UK). Quantitative real time PCR (7500 FAST thermocycler; Applied Biosystems) was used to examine the expression of D2 and D3 relative to stably expressed beta -2-microglobulin (B2M) [27, 28]. Reactions set up in duplicate were carried out in total volume of 20 µl comprising 10 µl FAST SYBR Green Master Mix (Applied Biosystems), 500nM forward primer, 500nM reverse primer, nuclease-free water and 1µl cDNA. The PCR programme ran at 95°C (20s) followed by 40 cycles of 95°C (3s), 60°C (30 s). Melt-curve analysis was

performed at 95°C - 60°C to confirm the presence of one single product. Two negative controls were included with each set of samples: (1) no RNA template; (2) RNA provided but no reverse transcription. The crossing point (CP) values were used for analysis, using a mathematical model for relative quantification developed by Pfaffl[29]. The relative expression ratio (*R*) of the target gene is calculated based on efficiency (*E*) and the *CP* deviation of an unknown sample versus a calibrator, and is expressed in comparison to a housekeeping gene [29, 30]. Primer sequences for D2 and D3 and for the housekeeping genes were as previously reported [31].

Immunohistochemistry: Immunohistochemical analysis was performed using the Dako Envision™ visualization system (Dako, Ely, UK) as previously described [23, 32]. D3 antibody (Abcam) was used at 0.5 µg/ml respectively, after determination of optimal dilutions (data not shown). Rabbit IgG was used in place of the specific antibodies as a negative control. Cerebral cortex was used as the positive control for the D3 antibody to verify specificity. A specific antibody for D2 in placentae could not be found and therefore not assayed.

D2 and D3 activity assays: The activities of specific deiodinase subtypes were estimated using methods previously described [33]. Briefly, the placental samples were homogenized in 10 vol 0.1 M phosphate (pH 7.2), 2 mM EDTA and 10 mM dithiothreitol (P100E2D1 buffer). Protein concentrations were estimated using the Bradford method [34]. D2 activity was determined by HPLC analysis of the production of radioactive iodide and T3 outer ring-labelled T_4, and D3 activity by HPLC analysis of the formation of radioactive T_2 and 3'-iodothyronine from outer ring-labeled T_3. Deiodination in the presence of placental

172 homogenate (~1 mg protein/ml) was corrected for non-enzymatic deiodination in the absence
173 of homogenate.
174
175 D2 activity assay: Incubations were carried out for 120 min at 37°C with 1 nM (10^5 cpm)
176 [3',5'-^{125}I]T_4 in the presence of 1 µM T_3 to block D3 and in the absence or presence of 100
177 nM T_4 to saturate D2, in 0.1 ml P100E2D10 buffer. Deiodinase activity was ascribed to D2 if
178 inhibited by excess unlabeled T_4.
179
180 D3 activity assay: Incubations were carried out for 60 min at 37°C with 1 nM (2×10^5 cpm)
181 [3'-^{125}I]T_3 in the absence or presence of 100 nM T_3 to saturate D3 in 0.1 ml P100D2D10
182 buffer. Deiodinase activity was ascribed to D3 if inhibited by excess unlabeled T_3. The
183 minimum detectable activity for deiodinase assays is (< 0.1fmol/min/mg protein) using this
184 methodology.
185
186 *Thyroid hormone assays:* Competitive immunoassays, using direct chemiluminescent
187 technology were used to measure FT3 and FT4 concentrations; a two-site sandwich
188 immunoassay for TSH concentrations in serum was used in the ADVIA Centaur system. All
189 serum samples were analysed in triplicate, with the inter- and intra-assays being less than 5%
190 and 10%, respectively. The pregnancy-specific reference ranges using this methodology for
191 the third trimester have been established in a recent study and are as follows, TSH: 0.5-4
192 mU/L; FT4: 8-14.5 pmol/L and FT3: 2.5-5.5 pmol/L [35].
193
194 *Selenium measurements:* Maternal and umbilical cord serum selenium concentrations on
195 these samples were determined by a Varian SpectrAA graphite furnace atomic absorption
196 spectrophotometer. These data have been previously reported [4].

197 *Statistical analysis:* All analyses were performed using SPSS for Windows, PASW18.0.
198 Summary data are presented as means ± SD or median [interquartile range] as appropriate for
199 their distribution, as determined by the Kolmogorov-Smirnov test. Within subject
200 comparisons were made using Friedman repeated measures ANOVA, between group analysis
201 using Mann-Whitney *U* or Student's *t* tests depending on the distribution and Spearman's
202 Rank tests were used for correlation analysis. The null hypothesis was rejected where $P < 0.05$.
203
204

Results

Subjects: Table 1 describes the demographic, obstetric, and pregnancy data of the 50 participants. Both pregnancy groups conceived spontaneously and carried singleton pregnancies. The normotensive pregnant women gave birth to infants weighing > 2500 g, delivered at 37 weeks or later.

Selenium concentrations: As previously reported [4], both maternal and umbilical cord serum selenium concentrations were significantly reduced in the pre-eclamptic compared with the normotensive group (Table 3).

Expression of mRNA for D2 and D3: Placental mRNA expression normalised to stably expressed B2M is reported as median value [interquartiles] and values are given for the middle sampling location; there was no effect of sampling site (P>0.3). D2 mRNA expression in the normotensive group was 0.23 [0.1-0.77] and in the pre-eclamptic placentae 0.38 [0.19-1.48]. There was no significant difference in expression between the two pregnancy groups (P=0.14). Placental D3 mRNA expression was also similarly expressed in both study groups (P=0.50); normotensive pregnancy, 2.8 [0.9-3.9] and pre-eclamptic pregnancy, 1.6 [0.6-4.2]. However, D3 expression was higher than D2 expression (Figure 1).

D3 immunohistochemistry: D3 immunostaining was localised to the syncytiotrophoblast with no difference in expression between the two groups (Figure 2).

D2 and D3 enzyme activities: D3 enzyme activity was identified in all placentae. Overall, when comparing groups, the enzyme activity did not differ between normotensive and pre-eclamptic women (P>0.05; Table 2). Placentae from pre-eclamptic women showed a

significant positive correlation between D3 activity and mRNA expression for all locations sampled (Figure 3; P<0.05); this correlation did not exist in the normotensive samples (P>0.05). However, a significant gradient in activity across the placental bed was evident only in the normotensive placentae; the highest activity was demonstrated nearest the cord (Friedman-Repeated Measures; P=0.034; Table 2). Mode of delivery or birthweights had no influence on D3 activities. To ensure observed differences in D3 activity and expression were not related to gestational age at delivery, we also compared these data with normotensive controls only for the 11 pre-eclamptic pregnancies who were delivered at ≥ 37 weeks' gestation. The comparison remained statistically significantly different (*P*>0.1 for both D3 activity and expression). D2 activity was undetectable in these samples.

Thyroid hormone results: One normotensive control woman had elevated maternal TSH concentrations and elevated umbilical FT3; these anomalies were not associated with clinically identified thyroid disorder and so were retained in the analysis.

No significant differences were seen between normotensive and pre-eclampsia samples for any of maternal TSH, FT4 or FT3 concentrations (Table 3; P>0.1for all). Umbilical venous TSH concentrations were significantly higher in the pre-eclamptic compared to the normotensive samples (Table 3; P<0.001) but umbilical venous FT4 and FT3 concentrations did not differ significantly. Mode of delivery had no influence on these levels. To ensure observed differences in umbilical venous TSH concentrations were not related to gestational age at delivery, we also compared these data with controls only for the 11 babies from pre-eclamptic pregnancies who were delivered at ≥ 37 weeks' gestation. The comparison remained statistically significantly different (*P* = 0.008). In addition, inverse relationships

were seen between umbilical venous TSH concentrations with our previously measured [4] umbilical venous TBARS concentration ($r = -0.60$; $R^2 = 0.40$; $P=0.03$) and plasma GPx activities ($r = -0.57$; $R^2 = 0.23$; $P=0.04$) in the pre-eclamptic samples only. Maternal TSH and FT4 were significantly lower and FT3 significantly higher than in matched umbilical samples (Table 3) in both normotensive and pre-eclamptic samples ($P < 0.05$ for all groups). There was no association between maternal or umbilical selenium concentration and simultaneously-measured TSH.

Deiodinases and Selenium: There was no direct association between mRNA and protein expression or activity of either deiodinase enzyme and maternal or fetal serum selenium concentrations.

Discussion

We have shown the presence of mRNA and protein D3 in term placenta, which is in agreement with previous studies [10-12]. Our original hypothesis was that there would be preferential utilisation of selenium by the iodothyronine deiodinases in pre-eclampsia. Our novel data support this hypothesis, since although there was no effect of pre-eclampsia on deiodinase mRNA or protein expression, there was a pre-eclampsia-related effect in relation to the selenoprotein D3 activity in the presence of significantly reduced serum selenium concentrations in both mother and fetus. Plasma FT_3 and FT_4 were similar in normotensive and pre-eclamptic women and their fetuses. The placental D2 activity, known to be at least 100 fold lower than D3 activity, was below the limits of detection in this study [9, 11].

Interestingly, a differential distribution of D3 activity was observed across the placental bed with highest activity near the cord insertion. However this gradient was seen only in placentae from normotensive pregnancies suggesting a possible blunting of D3 regulation in pre-eclampsia. We have previously reported gradients in enzyme activity across the placental bed in GPxs and Angiotensin converting enzyme [23, 36] as have others relating to gene expression [37] . This may relate to the lower tissue oxygenation at the periphery of the placenta, the central region being well-oxygenated owing to the direction of the maternal blood flow [38].

The regulation of D3 activity and expression is tightly controlled on a tissue-specific and even cellular level in a precise spatio-temporal manner [39]. D3 effects on thyroid hormone signalling occur via two routes according to oxygen availability [40]. When oxygen is adequate, D3 is moved from its site of synthesis in the endoplasmic reticulum to the Golgi

body and plasma membrane. However, when oxygen tension is low, D3 is redirected to the nucleus to be physically closer to the thyroid hormone receptor-mediated gene transcriptional control as demonstrated in human neuroblastoma cell line [40].

In colon cancer cells, D3 has been shown to be a direct transcriptional target for a complex including β-catenin [41] a key molecule in the Wnt signalling pathway which interacts with E-cadherin. Both mRNA and protein expression of β-catenin are down regulated in the term placenta in a similar fashion to E-cadherin expression [42]. In pre-eclampsia however, E-cadherin expression is elevated [43], whilst the expression of the zinc finger transcription factor Snail, which controls E-cadherin, is reduced in the placental periphery [44]. The perturbation of complex signalling networks within the placenta may contribute to the blunting of D3 regulation in pre-eclampsia.

The lack of direct relationship between selenium deficiency and thyroid function concurs with the current knowledge of the hierarchal control of selenoprotein expression in such deficient conditions [24]. Endocrine tissues are well adapted to maintain selenoprotein expression when selenium supply is limited and the deiodinases are maintained at the expense of GPxs, which are quickly lost [45, 46].

Our results support that maternal thyroid function, based on maternal TSH, FT3 and FT4 concentrations, did not alter in pre-eclamptic women compared to normotensive pregnant controls; this is consistent with others [47]. The thyroid hormones fell within the pregnancy reference ranges for this methodology [35] and were comparable with other studies [19, 48].

No significant differences were observed in umbilical venous FT3 and FT4 concentrations between the normotensive and pre-eclampsia groups; also consistent with other studies [49, 50]. However, a significant increase in TSH concentrations in the babies born to pre-eclamptic mothers compared to those babies born to normotensive women was observed. It has been suggested that the raised umbilical venous TSH concentrations in pre-eclampsia may reflect an adaptive response by the fetus to maximise iodide uptake, thereby maintaining normal levels of FT3 and FT4 [20]. Furthermore, the raised TSH concentrations may also be a consequence of increased hypoxia placed on these babies due to the inadequate placentation [49], although the exact mechanism has still to be elucidated. Interestingly, the novel inverse relationships seen between umbilical venous TSH concentrations with our previously measured umbilical venous TBARS concentration and plasma GPx activities in the pre-eclamptic samples only indicate that higher TSH concentrations in the pre-eclamptic fetuses are associated with increased the oxidative stress conditions.

This study illustrates that under the selenium deficiency seen in these pre-eclamptic women, the thyroid hormone homeostasis remains largely unchanged though there appear to be subtle differences in enzyme activity dependent on placental location, as well as in the translation between mRNA expression and protein activity. Future studies with larger cohorts will focus in the relationships of the fetal TSH with markers of both oxidative stress and the GPxs.

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Figure Legends

Figure 1: (A) D2 and (B) D3 mRNA expression across placental sampling sites normalised to housekeeper gene B2M. The values of both deiodinases are shown to the same scale to emphasise their relative differences in expression.

Figure 2: Placental D3 expression in (A) positive tissue control – cerebral cortex (B) negative control – IgG – placenta (C) Normotensive placenta (D) pre-eclamptic placenta; magnification x200 (arrows indicate syncytiotrophoblasts).

Figure 3: The relationship between the D3 mRNA expression and the D3 enzyme activity with both parameters tested across the 3 locations between A) normotensive and B) pre-eclamptic samples. Significant positive correlations were seen only in the pre-eclamptic samples (normotensive R2 - outer: 0.02; middle: 0.009; near: 0.003; P>0.05 for all, pre-eclampsia R2 – outer: 0.253, P=0.015; middle: 0.377, P=0.001; near: 0.253, P=0.034).