Contributton of Type II phospholipase A₂ to in vitro phospholipase A₂ enzymatic activity in human term placenta

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Abstract

Although phospholipase A₂ (PLA₂) enzymatic activities have been implicated in the regulation of phospholipid metabolism and eicosanoid formation in human gestational tissues, the role and contribution made by individual PLA₂ isozymes has not been established. The aim of this study, therefore, was to determine the contribution made by Type II PLA₂ to PLA₂ enzymatic activity present in human term placenta. The experimental paradigm used to establish the contribution made by Type II PLA₂ to total in vitro PLA₂ enzymatic activity present in placental extracts was to remove Type II PLA₂ by immunoaffinity extraction and then to quantify residual PLA₂ enzymatic activity. Before immunoaffinity extraction, Type II PLA₂ immunoactivity and total PLA₂ enzymatic activity present in placental extracts averaged 28·0 ± 10·0 ng/mg protein and 1040 ± 367 pmol/h per mg protein (n = 3) respectively. After solid-phase immunoaffinity batch extraction of placental extracts, immunoreactive Type II PLA₂ was not detectable by ELISA, and PLA₂ enzymatic activity was decreased by 82 ± 1% (P < 0·001). Residual (i.e. non-Type II) PLA₂ enzymatic activity was further characterised by Western blot analysis and enzyme activity assay. The data obtained are consistent with a contribution by both cytosolic PLA₂ and other secretory PLA₂ isozymes (i.e. non-Type II) to residual PLA₂ enzymatic activity. The results obtained in this study support the conclusion that Type II PLA₂ is quantitatively the primary PLA₂ isozyme that contributes to in vitro PLA₂ enzymatic activity present in extracts of human term placenta, accounting for at least 80% of total activity. These data further support the involvement of this extracellularly active isozyme in the regulation of placental phospholipid metabolism and eicosanoid formation during late gestation. Journal of Endocrinology (1998) 157, 25–31

Introduction

Phospholipases A₂ (PLA₂) represent a family of esterases that hydrolyse the sn-2-acyl ester bond of 1,2-diacyl-sn-3-phosphoglycerides, liberating equimolar amounts of 1-acyl-glycerophosphatide and free fatty acid (Slotboom et al 1978, Dennis et al. 1991). Obstetric interest in this signalling pathway has principally focused upon the role of phospholipases in liberating the fatty acid substrate, arachidonic acid, for the synthesis of eicosanoids, and in particular, prostaglandins. These phospholipid metabolites have been implicated in many aspects of pregnancy, fetal development and parturition (Thorburn & Rice 1988, 1990, Olson et al. 1992, López Bernal et al. 1993). In recent years, isozymes of PLA₂ that act intracellularly (cytosolic PLA₂; cPLA₂) or extracellularly (secretory PLA₂; Type II, Type IV and Type V) have been identified in human gestational tissues (Aitken et al. 1990, Bennett et al. 1993, Andersen et al. 1994, Chen et al. 1994, Buhl et al. 1995, Freed et al. 1997). While the functional role of cPLA₂ isozymes in generating glycerophospholipid-derived second messengers is consistent with our understanding of the action of these enzymes, the role of secretory PLA₂ isozymes that are released by human gestational tissues has yet to be clearly established (Rice 1995).

Although the expression of three secretory PLA₂ isozymes has been identified in human term gestational tissues, only one of these, Type II PLA₂, has been studied in any detail. The data obtained to date establish the presence of Type II PLA₂ mRNA (Aitken et al. 1992, Bennett et al. 1993, Freed et al. 1997) and immunoreactive protein (Aitken et al. 1993) in placenta, choriodicnda and amnion. Consistent with its extracellular site of action, Type II PLA₂ is secreted by human placenta, in vitro (Farrugia et al. 1997) and is present in maternal plasma during pregnancy (Pulkkinen et al. 1993). Consistent with the possible involvement of this isozyme in the onset and/or progression of labour and delivery, the concentration of immunoreactive (ir) Type II PLA₂ in maternal plasma increases in association with labour, both at term and preterm (Rice et al. 1992).

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Recently, the tissue-specific expression of Type II, Type IV and cPLA₂ mRNA transcripts was characterised in human term gestational tissues (Freed et al. 1997). In this study, Type II PLA₂ mRNA was reported to be the most abundant transcript present in human term placenta. These data support the hypothesis that Type II PLA₂ represents the major isozyme contributing to total PLA₂ enzymatic activity in this tissue. Previous studies have identified PLA₂ enzymatic activity in human term gestational tissues, but have not characterised the contribution made by the different PLA₂ isoforms present (Schultz et al. 1975, Grieves & Liggins 1976, Schwarz et al. 1976, Okazaki et al. 1978, López Bernal et al. 1992). Thus the aim of this study was to determine the contribution made by Type II PLA₂ to total PLA₂ enzymatic activity in human term placenta. Immunoaffinity batch extraction was utilised to remove Type II PLA₂ from placental extracts, and residual PLA₂ enzymatic activity was quantified and characterised.

Materials and Methods

Tissue collection and processing

Human placentae (n=3) were collected from women with uncomplicated singleton term (37–41 weeks of gestation) pregnancies at the Royal Women’s Hospital (Melbourne, Australia) in compliance with and approval of the research and ethics committees of the hospital. Placental tissues were collected at elective Caesarean section (i.e. before the onset of labour) and were transferred to the laboratory within 10 min of delivery. Attached fetal membranes were removed and discarded. Placental tissue was frozen in liquid nitrogen and stored at −40 °C.

Tissue extraction

Frozen tissues (10 g wet weight) were thawed and homogenised in 5 volumes of 1 M NaCl by 3 × 20 s bursts with a metal blade homogeniser (Jenke and Kunkel, Staufen, Germany). The homogenate was centrifuged at 900 g (model J2-MC centrifuge; Beckman, Palo Alto, CA, USA) for 10 min. The supernatant was collected and dialysed using 6–8 kDa cut-off dialysis tubing (Spectra/ Por; Spectrum Medical Industries Inc, Los Angeles, CA, USA) for 24 h at 4 °C against 4·5 litres 0·01 M Tris buffer (pH 8·0) containing 150 mM NaCl. The retentate was assayed for PLA₂ enzymatic activity, PLA₂ immunoactivity and protein content (see below). Data are expressed as percentage of pretreatment activity. The specificity of the monoclonal antibody was confirmed by Western blot analysis (Fig. 1).

Characterisation of residual PLA₂ enzyme activity

PLA₂ enzymatic activity remaining after two cycles of immunoaffinity batch extraction (residual PLA₂ enzymatic activity) was characterised by establishing the effects of known inhibitors of secretory PLA₂ enzymatic activity: dithiothreitol (DTT; 12·5 mM) or EGTA (12·5 mM) (Clark et al. 1990). Data are expressed as percentage of control activity.

PLA₂ ELISA

A non-competitive sandwich ELISA was used to measure PLA₂ content in extracted gestational tissue (Smith et al. 1990).
1992). Microtitre plates (96-well Maxisorp; Nunc, Roskilde, Denmark) were coated for 16 h at 4 °C with 100 µl purified monoclonal antibody raised against human recombinant Type II PLA₂ (9C1) at a dilution of 1:1000 in PBS. The wells were then blocked with 200 µl 1·0% (w/v) skimmed milk powder/0·1% (w/v) BSA in PBS for 3 h at 37 °C. The plates were then washed twice with 0·1% BSA in PBS. Aliquots (100 µl) of standard or diluted sample were added and incubated for 2 h at 37 °C. The plates were then washed. Purified monoclonal antibody 4A1 conjugated to alkaline phosphatase using glutaraldehyde was diluted 1:1000 in 0·1% BSA in PBS. Then 100 µl conjugated antibody was added to each well and incubated for 30 min at 37 °C. Plates were washed three times, followed by a further two washes with 20 mM carbonate buffer, pH 9·8. A 100 µl volume of the substrate p-nitrophenyl phosphate (1 mg/ml in carbonate buffer) was added to each well. The plates were placed at room temperature for at least 20 min and the absorbance at 405 nm was determined using a microplate reader (model 3350; Bio-Rad Laboratories, Richmond, CA, USA). A standard curve was generated by diluting purified recombinant PLA₂ in 0·1% BSA in PBS. Intra- and inter-assay coefficients of variation averaged 8·2 and 8·8% respectively.

**PLA₂ enzyme assay**

PLA₂ enzymatic activity was determined by the method of Green et al. (1991) as modified by Farrugia et al. (1993). Radiolabelled phospholipid 1-α-1-palmitoyl-2-[14C]arachidonylphosphatidylethanolamine (NEN Research Products, DuPont Australia, Sydney, NSW, Australia) was evaporated to dryness under nitrogen, resuspended in deoxycholate (in deionised water) and then diluted in Tris buffer (100 mM Tris, pH 8·0) containing 10 mM CaCl₂ and 300 mM NaCl before assay. Both substrate and samples were prewarmed to 37 °C before assay. Incubations were commenced by the addition of 25 µl phospholipid (0·22 nmol) to 25 µl diluted sample. Tubes were centrifuged briefly and then incubated for 1 h at 37 °C. Incubation mixture (30 µl) was then applied to a thin-layer chromatography plate (TLC) (Silica G; ICN, Costa Mesa, CA, USA). TLC plates were developed to a distance of 6 cm in chloroform/methanol/acetic acid (9:1:0·1, by volume). Radioactivity was localised by autoradiography (X-OMAT AR film; Kodak, Rochester, NY, USA), excised from the plates and quantified by liquid-scintillation spectrometry. Data are expressed as pmol phospholipid hydrolysed/h per mg protein (unless otherwise indicated). Intra- and inter-assay coefficients of variation averaged 9·8 and 14·3% respectively.

**SDS–PAGE and Western blot analysis**

The recovery of protein and ir Type II PLA₂ through tissue processing and extraction procedures was characterised by SDS–PAGE and Coomassie Brilliant Blue staining and by Western blot analysis. To negate the possibility of non-selective loss of PLA₂ during the procedure, the recovery of cPLA₂ was also monitored by Western blot analysis. Samples (20 µg protein) obtained at each stage of tissue processing and immunoaffinity extraction were diluted in loading buffer (125 mM Tris–HCl, pH 6·8; 4% SDS; 0·02% bromophenol blue; 20% glycerol), boiled for 5 min and then size fractionated on a 15% acrylamide gel under non-reducing conditions. Non-reducing conditions were used as the monoclonal antibody 4A1 recognises an epitope of Type II PLA₂ that is lost on reduction (data not shown). Molecular mass markers (Gibco–BRL, Grand Island, NY, USA) were run in parallel on all gels. Proteins were visualised by Coomassie Brilliant Blue staining or electrophoretically transferred to nitrocellulose membrane (NitroBind; Micron Separation Inc, Westboro, MA, USA), using a Hoefer semi-dry electrophoblotter (TE77, SemiPhor Transfer Unit) at 0·8 mA/cm² for 1·5 h. Nitrocellulose membranes were blocked with skimmed milk powder (0·5%, w/v) in Tris-bufered saline containing Tween 20 (0·05%, v/v; Sigma Chemical Co, St Louis, MO, USA) (TTBS) for 1 h at room temperature. Membranes were washed 3 × 10 min in TTBS and incubated with anti-Type II PLA₂ monoclonal antibody (4A1; 1:1000, v/v) or anti-cPLA₂ antibody (1:500, v/v; Genetics Institute, Cambridge, MA, USA) for 18 h at 4 °C. Membranes were washed (3 × 10 min) in TTBS and incubated with anti-mouse IgG–horseradish peroxidase conjugate (Silenus, Melbourne, Australia; for Type II PLA₂ development) or anti-rabbit IgG–horseradish peroxidase conjugate (Silenus; for cPLA₂ development) for 1·5 h at room temperature and then washed (3 × 10 min) in TTBS. Immunoreactivity was visualised using an enhanced chemiluminescence (ECL) procedure (Amersham, Arlington Heights, IL, USA).

**Protein assay**

The protein content of gestational tissues, homogenates and supernatants was determined by a protein dye-binding method (Bio-Rad Laboratories), utilising BSA as a reference standard (Bradford 1976).

**Statistical analyses**

Statistical computations were performed using a commercially available statistical analysis package (Statgraphics, STSC, Rockville, MD, USA). The homogeneity of data was tested using Bartlett’s test (Bartlett 1937). Subsequently, two sample comparisons were analysed using Student’s t-test, and three or more data sets were analysed using ANOVA. Statistical differences between individual group means were assessed using Student–Neuman–Keuls tests. Statistical significance was indicated by a P value of less than 0·05. Data are expressed as mean ± s.e.m.
Results

Placental tissue PLA₂ content

Before Type II PLA₂ immunoaffinity extraction, irType II PLA₂ and total in vitro PLA₂ enzymatic activity present in term placental tissue \((n=3)\) averaged \(28.0±10.0\, \text{ng/mg protein}\) and \(1040±376\, \text{pmol/h per mg protein}\) respectively.

SDS–PAGE and Western blot analysis

To determine the contribution made by Type II PLA₂ to total PLA₂ enzymatic activity, Type II PLA₂ was removed from placental extracts by solid-phase immunoaffinity batch extraction, and residual enzyme activity quantified and characterised. Initially, the effects of tissue processing and batch extraction on Type II PLA₂ and cPLA₂ immunoactivity and total protein were characterised by SDS–PAGE with Coomassie Brilliant Blue staining, and Western blot analysis respectively. A representative processing and extraction profile for proteins, PLA₂ irType II and cPLA₂ immunoactivity and total protein were characterised by SDS–PAGE with Coomassie Brilliant Blue staining, and Western blot analysis respectively. A representative processing and extraction profile for proteins, PLA₂ irType II and cPLA₂ immunoactivity and total protein were characterised by SDS–PAGE with Coomassie Brilliant Blue staining, and Western blot analysis respectively. A representative processing and extraction profile for proteins, PLA₂ irType II and cPLA₂ immunoactivity and total protein were characterised by SDS–PAGE with Coomassie Brilliant Blue staining, and Western blot analysis respectively.

Quantitative effects of immunoaffinity batch extraction

The quantitative effects of immunoaffinity batch extraction on Type II PLA₂ immunoactivity and total PLA₂ enzyme activity are presented in Fig. 3 as a percentage of activity present in the pretreatment sample. Treatment of placental extracts \((n=3)\) with Type II PLA₂ antibody coupled to agarose beads resulted in complete removal of Type II PLA₂ (i.e. not detected by ELISA) and a concomitant loss of \(82±1\%\) of PLA₂ enzymatic activity present throughout tissue processing and immunoaffinity batch extraction (Fig. 2b).

![Figure 2: SDS–PAGE of human recombinant Type II PLA₂ and placental extract (20 µg protein/lane).](image)

(a) Protein size fractionation and recovery before transfer to nitrocellulose and development using Coomassie Brilliant Blue. (c) Western blot analysis of irType II PLA₂ developed using ECL.

(b) Western blot analysis of immunoreactive cPLA₂ developed using ECL. Loading is as described above with the exception that lane 1 contains rabbit lung cPLA₂ standard and lane 8 is empty.

![Figure 3: Immunoaffinity extraction of Type II PLA₂ from human term placental extracts (n=3) using control monoclonal antibody–agarose conjugate (Anti-MSA) or anti-Type II PLA₂ conjugate (Anti-PLA₂). Placental extracts were subjected to two cycles (1st) and (2nd) of immunoaffinity extraction and assayed for enzymatic activity and Type II PLA₂ immunoactivity. Data are presented as percentage of pretreatment activity and represent the mean ± s.e.m. (n=3). nd, not detectable.](image)
phospholipid metabolism and the formation of second messengers during pregnancy and at the time of labour and delivery (Rice 1996). To date, the contribution made by individual PLA₂ isozymes to PLA₂ enzymatic activity has not been established. Thus, the aim of this study was to establish the contribution made by Type II PLA₂ to total PLA₂ enzymatic activity present in human term placenta when assayed under in vitro conditions.

In this study, high ionic strength (HIS) extraction medium (i.e. 1 M NaCl) was used to facilitate the recovery of both secretory and cytosolic PLA₂ isozymes from placental homogenates. We have previously demonstrated that the use of such medium results in a 10- to 20-fold greater recovery of PLA₂ compared with the conventionally used low ionic strength extraction media (such as 0·16 M sucrose, Tris-buffered saline or PBS) (Aitken et al. 1993, Farrugia et al. 1993). Furthermore, HIS medium effectively dissociates calcium-dependent cPLA₂–cell membrane binding (Clark et al. 1990), based on the observation that the inclusion of chelating agent (3 mM EGTA) in HIS medium does not result in increased recovery of PLA₂ activity (Farrugia et al. 1993). The presence of both secretory and cytosolic PLA₂ in HIS extracts of placenta was confirmed by Western blot analysis (Fig. 1). PLA₂ enzymatic activity present in HIS extracts was assayed under conditions optimal for both secretory and cytosolic PLA₂ (i.e. the presence of millimolar calcium concentrations, neutral–alkaline pH and an sn-2-arachidonoylphospholipid substrate (Rice 1995)) and was consistent with previously reported values (Aitken et al. 1993, Farrugia et al. 1993).

The treatment of placental extracts with Type II PLA₂ monoclonal antibody coupled to agarose beads resulted in complete removal of irType II PLA₂, as determined by ELISA. The specificity of the antibody used to remove irType II PLA₂ from placental extracts was confirmed by Western blot analysis. After SDS–PAGE fractionation of placental proteins, the antibody bound to a protein band that co-migrated with Type II PLA₂ standard, and no significant cross-reactivity with other placental proteins was observed (Fig. 1). Using this antibody, immunoaffinity extraction of Type II PLA₂ was associated with an 82% decrease in PLA₂ enzymatic activity (Fig. 3). No significant loss of immunoactivity and enzymatic activity was associated with parallel extractions in which a control monoclonal antibody (MSA) was used.

The treatment of placental extracts with Type II PLA₂ by two cycles of immunoaffinity batch extraction, residual PLA₂ enzymatic activity was still detectable. The presence of immuno-reactive cPLA₂ in immunoaffinity-treated extracts was confirmed by Western blot analysis (Fig. 2), and thus this isozyme must contribute to the observed residual enzymatic activity. To characterise the residual activity further, the effects of known inhibitors of secretory PLA₂ isozymes were determined. The data obtained using DTT and EGTA treatment, while not unequivocal, are suggestive

Characterisation of residual PLA₂ enzymatic activity

The effects of DTT and EGTA on PLA₂ enzymatic activity remaining after two cycles of immunoaffinity extraction were presented in Fig. 4. Comparative data (controls) for human recombinant Type II PLA₂ and cPLA₂ standards are also presented. Data are presented as percentage of pretreatment enzymatic activity (n=3). Pretreatment placental extract, human recombinant Type II PLA₂ and residual PLA₂ enzymatic activity were significantly inhibited in the presence of reducing agent (12·5 mM DTT; P<0·05). Pretreatment placental extract displayed the greatest sensitivity to DTT inhibition with 89 ± 3% of activity inhibited (P<0·001) followed by human recombinant Type II PLA₂ (64 ± 1%, P<0·01) and residual PLA₂ (30 ± 9%, P<0·05). cPLA₂ standard isolated from rabbit lung was unaffected by DTT treatment. The enzymatic activity of all preparations was suppressed by treatment with calcium-chelating agent (EGTA, 12·5 mM). Pretreatment placental extract and human recombinant Type II PLA₂ displayed the greatest sensitivity to EGTA inhibition with 95 ± 1% and 94 ± 1% of activity inhibited (P<0·01), followed by residual PLA₂ (80 ± 3%, P<0·01) and cPLA₂ (42 ± 7%, P<0·01).

Discussion

Multiple isozymes of PLA₂ have been identified in human term placenta and may contribute to cell membrane
that both cytoplasmic and secretory PLA₂ isozymes contribute to the residual activity. The residual activity displayed a response to inhibitors that fell between that of recombinant Type II PLA₂ and cPLA₂ (Fig. 4). It is not possible from the data obtained in this study to determine whether the secretory PLA₂ component of residual activity (i.e. DTT-sensitive) represents a non-Type II PLA₂ (e.g. Type IV PLA₂ which has been identified in human placenta; Freed et al. 1997) or the presence of Type II PLA₂ at a concentration that is below the limit of detection of the ELISA (5 ng/ml). The development of further immunological probes will be required to resolve this issue. The data obtained in this study, however, unequivocally established Type II PLA₂ as a quantitatively important PLA₂ isozyme present in human term placenta. While the precise physiological role of placentalf PLA₂ remains to be elucidated, this isozyme is released from term placenta (Farrugia et al. 1997) and is present in maternal plasma during pregnancy (Rice et al. 1992). The concentration of rType II PLA₂ in maternal plasma increases at the time of labour and delivery both at term and preterm. The release of PLA₂ from placenta (and possibly other gestational tissue, including amnion and choriodicervia; Aitken et al. 1993) may contribute to increased release of arachidonic acid and its subsequent conversion to uterotonic eicosanoids that may facilitate labour and delivery. The efficacy of exogenous PLA₂ and arachidonic acid in stimulating eicosanoid formation has been previously established (Gu & Rice 1991, Hara et al. 1991). In addition to a role in the formation of eicosanoids during pregnancy, secretory PLA₂ isozymes have been proposed as regulators of the cell-surface expression of aminophospholipids (Rice 1996). The cell-surface expression of aminophospholipids has been implicated in cell membrane fusion (Connor et al. 1989), the assembly of cell-surface clotting factors (Gilbert & Drinkwater 1993) and cell recognition (Herrmann & Devaux 1990). Type II PLA₂ displays a substrate preference for aminophospholipids and thus may play an important role in the placenta in regulating the composition of the exoplasmic leaflet of the cell membrane bilayer. By hydrolysing aminophospholipids and limiting the expression of negatively charged phospholipids on the cell surface, this isozyme may help to prevent clot formation (for example, in the intervillous space) and affect the rate of cytotrophoblast syncytial formation. In summary, the data obtained in this study support the conclusion that Type II PLA₂ is quantitatively the primary PLA₂ isozyme present in human term placenta. Under the experimental conditions used in this study, Type II PLA₂ accounted for at least 80% of total PLA₂ enzymatic activity in the placenta. Although this isozyme has been demonstrated to be in abundance in placental homogenate, it is a secreted isozyme and active in the extracellular compartment. Once released, Type II PLA₂ may contribute to placental phospholipid metabolism and eicosanoid formation. Within the intracellular compartment, cPLA₂ contributes to the metabolism of phospholipids and the liberation of fatty acid substrate for eicosanoid formation. The relative contributions of these pathways to eicosanoid formation remain to be established.

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