Effect of bacterial endotoxin on the in vitro release of Type II phospholipase-A₂ and prostaglandin E₂ from human placenta

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Abstract

The aim of this study was to establish the effect of bacterial endotoxin lipopolysaccharide (LPS) on the release of Type II phospholipase-A₂ (PLA₂) and prostaglandin E₂ (PGE₂) from late-pregnant human placental tissue incubated in vitro. Under basal conditions, immunoreactive Type II PLA₂ and PGE₂ were released from tissue explants in a time-dependent manner (up to 24 h, ANOVA, P<0·0001, n=6). The release of these mediators was not associated with a loss of cell membrane integrity, as indicated by concentrations of the intracellular enzyme, lactate dehydrogenase (LDH), in the incubation medium. Incubation of explants in the presence of LPS (0·001–100 µg LPS/ml) significantly decreased PLA₂ tissue content (P<0·02, n=6) and increased the accumulation of PLA₂ and PGE₂ in the incubation medium (P<0·0001, n=6). The data obtained in this study indicated that Type II PLA₂ and PGE₂ are released from term placenta under basal conditions and that LPS stimulated their release. The associated decrease in PLA₂ tissue content is consistent with the hypothesis that LPS induces the release of stored PLA₂. This study identifies one pathway by which products of bacterial infection may induce the release of a pro-inflammatory, extracellularly active PLA₂ from intrauterine tissues that may promote the formation of phospholipid metabolites involved in the process of labour and delivery (e.g. the prostaglandins).


Introduction

Although the aetiology of preterm labour remains equivocal, reproductive tract bacterial infection has been implicated as a significant risk factor (Daikoku et al. 1982, Gravett et al. 1986, Quinn et al. 1987, Hillier et al. 1988, Romero et al. 1988, 1989). One of the proposed underlying biochemical pathways by which bacterial infection may initiate preterm labour is via bacterial endotoxin-induced formation of pro-inflammatory mediators that directly or indirectly promote myometrial responsiveness or activity. Bacterial endotoxins, such as lipopolysaccharide (LPS), induce inflammatory responses in human gestational tissues, resulting in increased release of pro-inflammatory cytokines, such as cytokine interleukin-1 (IL-1) (Taniguchi et al. 1991, Kauma et al. 1992) and phospholipid metabolites such as prostanooids (Romero et al. 1988, Casey et al. 1990, Mitchell et al. 1990, Lundin-Schiller & Mitchell 1991, Nguyen et al. 1994).

The mechanisms by which LPS affects the formation of phospholipid metabolites in human gestational tissues, and in particular, the primary phospholipase isozyme (Type II phospholipase-A₂ (PLA₂)) present in human placenta have yet to be established. Type II PLA₂ accounts for greater than 80% of total tissue PLA₂ enzyme activity present in term placenta (Rice et al. 1998). It is released from gestational tissue and is thought to act on phospholipids in the exoplasmic leaflet of the cell membrane (Farrugia et al. 1997, Rice et al. 1998). Type II PLA₂ messenger RNA, immunoreactive content and enzymatic activity have been identified in human gestational tissues, including: amnion, chorioamnion and placenta (Aitken et al. 1990, 1992, Farrugia et al. 1993, Andersen et al. 1994). Although the mechanisms that regulate the release of this isozyme from gestational tissues have yet to be determined, increased release of Type II PLA₂ may contribute to the well-characterised labour-associated increase in gestational tissue phospholipid metabolism and prostanooid formation (Embrey 1971, Kierse et al. 1977a,b, Sellers et al. 1981) and to the increased concentrations of Type II PLA₂ in maternal plasma that occur at this time (Rice et al. 1992). In non-gestational tissues, immunohistochemical studies have demonstrated that Type II PLA₂ is located in the secretory granules (Chock et al. 1994). Thus, increased exocytotic release of Type II PLA₂ may represent one process involved in these labour-associated changes.

The aim of this study was to determine the effects of LPS on placental Type II PLA₂ tissue content and release...
in vitro and the concomitant release of the prostanoid prostaglandin E2 (PGE₂). The hypothesis to be tested was that LPS-treatment would increase the release of Type II PLA₂ and PGE₂ from placental explants. The experimental model utilised to evaluate the effects of LPS was a well-established placental explant incubation system (Farrugia et al. 1997).

Material and Methods

Reagents

The following reagents were obtained from BDH Chemicals Australia (Melbourne, Victoria, Australia): di-potassium hydrogen orthophosphate, potassium di-hydrogen orthophosphate, di-sodium hydrogen phosphate, sodium dihydrogen orthophosphate, sodium chloride, sodium azide, calcium chloride, gelatin (type A), activated charcoal, dextran-T70, ammonium sulphate, Tween-20, glutaraldehyde, deoxycholate, p-nitrophenyl phosphate, sodium carbonate, sodium hydrogen carbonate, magnesium chloride, and reagent grade chloroform, glacial acetic acid, and methanol. LPS (phenol extracted, Escherichia coli, serotype 026:B6), Type II pyruvic acid (dimer free), β-NADH (disodium salt), BSA (RIA grade), and HCl were purchased from Sigma Chemical Co. (St Louis, MO, USA). Alkaline phosphatase (calf intestine grade 1) was purchased from Boehringer-Mannheim Australia (Sydney, NSW, Australia). Prostaglandin (PG) standards, PGE₂, were from Sigma. PGE₂ radiolabel (5·6·8·11·12·14·15(n)-[3H]PGE₂: 5·9–8·9 Tbq/mmol) was obtained from Amersham Australia (Sydney, NSW, Australia). The scintillation used was Optiphase ‘HiSafe’ 3 from Wallac Scintillation Products (Turku, Finland). RPMI 1640 (phenol red free), penicillin G and streptomycin were obtained from Gibco Laboratories (Grand Island, NY, USA). Rabbit polyclonal antiserum raised against PGE₂-porcine thyroglobulin conjugate was provided by Dr P Christensen, Department of Experimental Medicine, The Panum Institute, Copenhagen, Denmark. Type II human PLA₂ monoclonal antibodies (4A1 and 9C1) were prepared by Bioquest (Sydney, NSW, Australia) and recombinant expressed human Type II PLA₂ standard was isolated from Chinese hamster ovary cell line stably transfected with human Type II PLA₂ under a metallothioneine promoter (Smith et al. 1992).

Buffers and extraction medium

The following buffers and extraction media were utilised: PBS (10 mmol/l sodium dihydrogen phosphate, 10 mmol/l di-sodium hydrogen phosphate and 100 mmol/l sodium chloride, pH 7·4), carbonate buffer (20 mmol/l sodium carbonate, 35 mmol/l sodium hydrogen carbonate and 4 mmol/l magnesium chloride, pH 9·8), PLA₂ ELISA washing buffer (135 mmol/l sodium chloride, 3 mmol/l potassium chloride, 2 mmol/l sodium dihydrogen phosphate, 15 mmol/l di-sodium hydrogen phosphate, 0·5 ml/l Tween-20 and 1 g/l BSA, pH 7·4), tissue extraction solution (1 M NaCl), 300 mmol/l sodium chloride, pH 8·0), PGE₂ assay buffer (10 mmol/l di-potassium hydrogen orthophosphate, 10 mmol/l potassium dihydrogen orthophosphate, 0·1% (w/v), 15 mmol/l sodium azide), lactate dehydrogenase (LDH) phosphate buffer (40 mmol/l potassium dihydrogen orthophosphate, 5 mmol/l di-potassium hydrogen orthophosphate, pH 7·0), β-NADH (10 mg/ml), LDH solution A (350 µl pyruvic acid (2·5 mg/ml) are added to 9·65 ml LDH phosphate buffer).

Tissue collection and preparation of explants

Human placenta was obtained (with institutional Research and Ethics Committee approval) at elective Caesarean section (i.e. from women before the onset of labour with uncomplicated pregnancies). Seven placentae were used in the experiments on the effects of LPS dose on placental PLA₂ tissue content and a further six placentae were required to investigate the effect of 1 µg/ml LPS on Type II PLA₂. Tissues were received at the laboratory within 10–15 min of delivery. A section of placenta was placed in ice-cold RPMI 1640. The tissue was teased apart using blunt dissection and placed in RPMI 1640 at 37°C in a humidified atmosphere of carbogen (95% O₂ and 5% CO₂) for 1 h. Explants were then blotted dry on sterile filter paper and transferred to 12-well tissue culture plates (Flow Laboratories, McLean, VA, USA) (150–250 mg wet weight/well): the explants were incubated in 2 ml RPMI 1640 or RPMI 1640/LPS containing penicillin G (100 U/ml) and streptomycin (100 µg/ml) for up to 8 h. Media were collected at 1, 2, 4 and 8 h; at each time point 2 ml appropriate incubation medium were completely replaced. The LPS dose experiments were incubated for 24 h with no medium replacement.

Tissue processing

Tissues were homogenised in 1 M NaCl (1:5; w:v) for 20 s using a metal-blade tissue homogeniser (Ultra-turrax, T25 and S25N 8G dispersing tool; Jenke and Kunkel GMBH and Co., Staufen, Germany). The homogenate was centrifuged at 2500 r.p.m. for 10 min at 4°C. The supernate was collected and stored at −20°C until assayed.

Lactate dehydrogenase assay

To negate possible confounding effects of experimental treatments on cell membrane integrity, the release of the intracellular enzyme LDH into incubation medium was determined. A total of 10 µl β-NADH were added to
50 µl incubation medium, and 12 µl mixture were transferred to a well in a microtiter plate. A total of 100 µl LDH solution A were then added to each well and immediately mixed in the microplate reader. The microplate reader was programmed to determine absorbance at 340 nm every minute (with 10 s mixing before each reading) for a 10-min duration. Enzyme activity was determined by the rate of change of absorbance, calculated using an extinction coefficient of 6.22 and expressed as a specific activity (µg/mg protein).

Prostaglandin E2 radioimmunoassay

The concentration of PGE2 in incubation medium was assayed as described previously (Rice et al. 1988) and fully described by Nguyen et al. (1994). The sensitivity of the assay averaged 0.02 pmol PGE2/tube. The intra- and interassay coefficients of variation were 6.7 and 13.4% respectively. Neither RPMI 1640 nor LPS at concentrations up to 1 µg/ml interfered with the assay.

Type II PLA2 ELISA

Type II PLA2 immunoreactivity was quantified by a non-competitive sandwich ELISA using two monoclonal antibodies (9C1 and 4A1) raised against recombinant human Type II PLA2 as described previously by Farrugia et al. (1993). The assay was modified by adding an extra wash at each appropriate step. The limit of detection of the assay (defined as two standard deviations from the zero standard) was 0.3 ng/well. The intra- and interassay coefficients of variation determined over ten assays were 8.2 and 8.8% respectively.

Protein assay

The protein content of gestational tissues, homogenates and supernates was determined by the protein dye-binding method described by Bradford (1976) using bovine serum albumin as a reference standard.

Statistical analyses

Statistical computations were performed using a commercially available statistical analysis package (Statgraphics, STSC, Maryland, USA). The homogeneity of data was tested using Bartlett’s test (Bartlett 1937). Where Bartlett’s test deviated from homogeneity (P<0.05), the data were transformed and homogeneity of variance confirmed. Significance between individual points was determined using the Newman–Keul’s test. Subsequently, two sample comparisons were analysed using Student’s t-test, and three or more data sets were analysed using analysis of variance (ANOVA). Statistical significance was indicated by a P value of less than 0.05. Data are expressed as means ± S.E.M.

Results

Phenol red, at a concentration of 0.01 mM (a concentration routinely used in incubation medium) was found to suppress LPS-induced PLA2 accumulation in incubation medium by 70% compared with incubations free of phenol red. All experiments were therefore carried out in phenol red-free RPMI.

Experiment 1: effect of LPS on placental Type II PLA2 tissue content

To determine the effect of LPS on Type II PLA2 tissue content, placental explants were incubated in the absence (control) and presence of increasing concentrations of LPS (0.001–100 µg LPS/ml) for 24 h (Fig. 1). LPS-treatment was associated with a significant decrease in placental Type II PLA2 content (P<0.02). Multiple range analysis indicated significance, different letters denote statistical significance (P<0.05) and (*) denotes significant change from control (i.e. in the absence of LPS, P<0.05).
of cell membrane integrity) was identified. In all subsequent experiments, LPS was used at a concentration of 1·0 µg/ml for 8 h.

**Experiment 2: effect of LPS on the release of Type II PLA$_2$ and PGE$_2$ from placental explants**

To determine the effect of LPS on the concomitant release of Type II PLA$_2$ and PGE$_2$, explants were incubated in the absence (control) or presence of 1·0 µg LPS/ml for up to 8 h. Under basal conditions (i.e. in the absence of LPS), the concentrations of immunoreactive Type II PLA$_2$ and PGE$_2$ in incubation medium increased in a time-dependent manner over 8 h (ANOVA, $P<0·001; n=6$). In the presence of LPS, the release of both Type II PLA$_2$ and PGE$_2$ into the incubation medium was significantly increased ($P<0·05; n=6$). A typical plot is shown in Fig. 2 and group data ($n=6$) are summarised and presented as (treatment–control) responses in Fig. 3. No significant effect of incubation time on the accumulation of LDH in incubation medium was identified.

The release of PGE$_2$ from explants was significantly correlated with Type II PLA$_2$ release under basal ($PGE_2=5·7$ PLA$_2=60·5$, $r^2=0·90$, $n=6$) and LPS-stimulated conditions ($PGE_2=4·1$ PLA$_2=40·4$, $r^2=0·95$, $n=6$).

**Discussion**

The aim of this study was to determine the effect of bacterial endotoxin (LPS) on the concomitant release of a secreted, extracellularly active PLA$_2$ isozyme (Type II PLA$_2$) and the prostanoid, PGE$_2$, from term placenta. The data obtained are consistent with the hypothesis that Type II PLA$_2$ is released by term placenta and that the release of isozyme is enhanced by bacterial endotoxin. PGE$_2$ release from placental explants paralleled, and was significantly correlated with, that of Type II PLA$_2$.

Under basal conditions, immunoreactive Type II PLA$_2$ accumulated in incubation medium in a time-dependent manner ($P<0·0001; n=6$). The increase in the concentration of phospholipase in incubation medium was not associated with a loss of cell membrane integrity, as indicated by the concentrations of the intracellular enzyme, LDH, in the incubation medium. In the presence of LPS, Type II PLA$_2$, placental tissue content decreased and incubation medium concentration increased significantly when compared with control incubations ($P<0·02$, $n=6$).

Previously, Type II PLA$_2$ has been localised, using immunohistochemistry and in situ hybridisation, to trophoblast cells, vascular smooth muscle and mesenchymal elements of the placenta (Aitken et al. 1996). In non-gestation tissues, Type II PLA$_2$ has been identified in secretory granules and is released from tissues via a process of exocytosis (Rosenthal et al. 1995). In these tissues, the release of Type II PLA$_2$ has been implicated in the development and progression of inflammatory reactions in response to infection (Vadas & Pruzanski 1986). By an analogous process, secretory Type II PLA$_2$ may be induced in human intrauterine tissues in response to infection (e.g. chorioamnionitis) and, in particular, in response to bacterial endotoxin (LPS), resulting in the formation of uterotonic phospholipid metabolites and the premature activation of labour. In support of this proposal, previous studies have identified an association between lower genital tract bacterial infection and human preterm labour (Daikoku et al. 1982, Gravett et al. 1986, Quinn et al. 1987, Hillier et al. 1988, Romero et al. 1988, 1989).
addition, bacterial endotoxins from gram-negative bacteria have been reported to stimulate the synthesis and release of PGs (e.g. PGE_2 and PGF_2alpha) by human gestational tissues (Quinn et al. 1987, Hillier et al. 1988, Romero et al. 1989). Thus, LPS may induce the release of Type II PLA_2 containing secretory granules from placenta tissue. Once released, this phospholipase isozyme may act locally to promote phospholipid hydrolysis and the liberation of substrate for prostanoid synthesis.

In conclusion, this study has characterised the co-release of Type II PLA_2 and PGE_2 from human placenta, under basal and LPS-stimulated conditions. The release of these mediators may contribute to increased intrauterine phospholipid metabolism and the initiation and/or maintenance of labour at term, or preterm in association with reproductive tract bacterial infection. Phenol red, a common additive to tissue culture media, displayed an anti-inflammatory action in suppressing LPS-induced release of Type II PLA_2.

Acknowledgements

The work described in the manuscript was supported by a project grant from the National Health and Medical Research Council of Australia (G E R), the 3AW Community Service Trust (G E R, S P B), the Royal Australian College of Obstetricians and Gynaecologists (G E R, S P B). G E R is in receipt of a National Health and Medical Research Council of Australia Principal Research Fellowship. The authors gratefully acknowledge the assistance of clinical research nurses Madonna Grehan and Linda Horton and the obstetric and midwifery staff of the Royal Women’s Hospital for their cooperation.

References


Received 8 June 1998
Revised manuscript received 10 September 1998
Accepted 8 October 1998