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Determination of Prostaglandin Profiles in LPS-Challenged Guinea Pig Spleen

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Abstract

We previously reported that splenic extract from lipopolysaccharide (LPS)-challenged guinea pigs inhibits the exaggerated febrile response of splenectomized guinea pigs, suggesting that the spleen generates an inhibitory factor. Earlier results indicate that the factor is a lipid. In an effort to identify this factor, lipid fractions, isolated from splenic extracts of control and LPS-challenged guinea pigs were analyzed with emphasis on identifying and quantifying prostanoids, which according to current knowledge are the likely bioactive factors. Prostaglandins have been extensively implicated in central and peripheral thermoregulation, and thus these lipids were targeted for characterization in the spleen. Analysis was done on the splenic extracts using solid-phase extraction, analytical and preparative thin layer chromatography (TLC) and high performance liquid chromatography-mass spectrometry (HPLC-MS/MS). Four prostaglandins (PGs, 6-keto-PGF_{1α}, PGF_{2α}, PGE₂, and PGD₂) were identified and quantified. Our data shows that these PG levels are doubled in LPS-treated guinea pig spleen compared with the control group. The methods used in this investigation to characterize PG in the spleen offer significant advantages over immunoassays previously used to identify and quantify PG in the spleen and other biological tissues. These methods will be utilized in further research needed to definitively characterize the role of splenic-derived PG in modulation of the febrile response induced by LPS.

Keywords

Prostaglandins; Fever; Lipopolysaccharide; HPLC-MS/MS; TLC

Introduction

Fever is a common clinical sign encountered in various diseases, especially during infections. The mediators, their interactions and the mechanisms involved in fever are complicated and have been rigorously investigated for decades [Atkins, 1960; Bartfai, et. al. 2010]. Previously, we proposed a novel, unrecognized, endogenous anti-hyperpyretic mechanism that may limit exaggerated fevers [Feleder et. al. 2003; Feleder et al., 2005]. This hypothesis was based on data, showing for the first time, that the spleen may modulate the febrile response to LPS. We reported that splenectomy increased both the magnitude of the core temperature rise and the Kupffer cell uptake of LPS of guinea pigs in response to systemically administered LPS, suggesting that the spleen may normally modulate endotoxic

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fever by releasing a factor into the portal vein that serves to limit LPS uptake by Kupffer cells [Feleder et. al, 2003]. We also found that intravenous injection of ultra-filtered (MW cutoff: 10kDa) splenic tissue extracts attenuates LPS-induced fever, and the splenic inhibitory factor may exist in the protein-free part of the spleen extract. These findings suggest that this factor is a lipid, perhaps a prostanoid [Feleder et. al, 2005]. Prostanoids are produced by the spleen, are released into the splenic vein, and thereby affect Kupffer cell activity. Several lines of evidence suggest that our splenic inhibitory factor contains one or more prostanoids. First, macrophages in the marginal zone of the spleen secrete PGE₂, in response to LPS and gram-negative bacteria [Ge et. al, 1997] and consequently, directly expose Kupffer cells to splenic PGE₂ via the portal vein [Marshall et. al, 1993; Billiar et. al, 1988]. PGE₂ is also released into the splenic vein during hemorrhagic and septic shock [Roland et. al, 1999; Tanaka et. al, 1996]. PGD₂ is also produced by the spleen and modulates the response of Kupffer cells and hepatocytes (e.g., PGD₂ binds to Kupffer cells) and it has been shown that it participates in the inflammatory response by inhibiting Kupffer cells' activity [Kuiper et. al, 1989; Urade et al, 1987; Urade et al., 1989]. Other prostanoids that have been implicated in the regulation by the liver of host defense reactions including 6-keto-PGF_{1α} and thromboxane (TX)B₂. Both prostanoids are released by splenic macrophages, and a significant increase in the portal blood levels of these prostanoids was reported in LPS-treated rats [Wu et. al, 1993; Kitagawa, et al, 1999]. Their concentration levels in the splenic vein were also drastically higher than in the mesenteric vein [Wernze et. al., 1986], suggesting that they were "utilized" by the liver, and thus probably contributed to the modulation of the liver by the spleen. Hence, either one of these prostanoids or a combination of prostanoids could be our putative factor.

Therefore, we used a sequence of analytical and preparative chromatographic methods, coupled with mass spectrometry, to identify a profile of prostaglandins isolated from the spleen of LPS-challenged guinea pigs that might play a role in modulation of the febrile response to infection. Preliminary analysis of the lipid components of protein-free splenic extract (MW cut-off 10kD) was done using thin layer chromatography (TLC). Isolation of the total lipid components from the spleen extract, and subsequent analytical TLC analysis showed the presence of lipids that strongly correlate with PGF_{2α}, PGE₂/PGD₂ and TXB₂. This total lipid fraction was shown to exhibit an antipyretic effect when fever is induced with LPS in splenectomized guinea pigs. Correlation of this data with the preponderance of evidence that PGs play a prominent role in fever, prompted us to develop a method to isolate and characterize PGs from the guinea pig spleen. Thus, we prepared splenic extract from guinea pigs receiving LPS or pyrogen-free saline, using solid phase extraction (SPE) followed by qualitative and quantitative identification of PGs using LC-MS/MS. This method allowed simultaneous qualitative and quantitative analysis of four prostaglandins and offers advantages over immunoassays (ELISA or RIA) that have previously been used to identify PG in biological fluids and tissues.

1. Experimental

2.1 Chemicals and materials

The prostaglandin standards including 6-keto-PGF_{1α}, PGF_{2α}, PGE₂, PGD₂, PGJ₂ and phosphatidylcholine, phosphatidylethanolamine, cholesterol, cholesterol linoleate, sphingomyelin, TXB₂, L-α-lysophosphatidylcholine, L-α-lysophosphatidylethanolamine were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile, methanol, ethanol, methyl formate, and chloroform were purchased from Fisher Scientific (Waltham, MA, USA). Formic acid, acetic acid, phosphomolybdic acid and LPS (from *Escherichia coli* 055:B5) were also obtained from Sigma-Aldrich (St. Louis, MO, USA). Other materials were obtained from vendors as indicated in the text.

2.2 Animals

Male Hartley guinea pigs (300–350g, Charles River Laboratories, Wilmington, MA) were used in these experiments. Animals were housed under standard conditions and provided with food (Agway Prolab guinea pig diet) and water ad libitum. After a 1 week acclimation period, guinea pigs were separated into two groups, 8 animals in each group. LPS (ip, 8 μ g/kg, 15 min) was administered to guinea pigs in the treatment group and the control group received pyrogen-free saline (PFS). After the administration of LPS or PFS, guinea pigs were deeply anesthetized (ketamine-xylazine) and spleens were quickly removed and weighed under aseptic conditions [Feleder et. al., 2005]. The animal protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3 Preparation of Spleen Extracts

Splenic tissues were then homogenized in ice cold PBS (5ml, pyrogen-free phosphate saline buffer solution) and centrifuged at 4,500g, 4°C for 20 min. The supernatant was centrifuged at 14,000 g, 4°C for a further 10 min. The resulting supernatant was ultra-filtered using a Centriprep-10 centrifugal concentrator (10kD molecular weight cut-off (Amicon Inc., Beverly, MA) to remove high molecular weight proteins. The spleen extract was stored at –80°C.

2.4 Protein Measurement in Spleen Extract

The concentration of proteins in the spleen extracts was measured using the RC-DC protein assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers specifications. A standard curve for the protein assay was generated with bovine serum albumin (BSA) in 12% SDS. Duplicate splenic samples were used for protein concentration measurement. Protein concentrations were measured in splenic samples prior and after addition of 20% TCA (1:1 v/v) to precipitate residual low molecular weight proteins. The protein concentration in these samples was obtained by interpolation from the linear portion of the protein standard curve.

2.5 Folch Extraction of Lipid Components of Spleen Extract

Spleen extract for TLC analysis was thawed in an ice bath and subjected to Folch extraction (2:1, chloroform (CHCl₃): methanol (MeOH)) to isolate lipid components [24]. Spleen extract was vigorously stirred with Folch solvent (3ml) in a glass vial, under N₂ atmosphere at room temperature for 15 minutes. Stirring was discontinued and the layers were allowed to separate. The top, aqueous layer was drawn off with a pipette and transferred to a second, clean, glass vial. The remaining organic layer in the vial was temporarily set aside. An additional volume of Folch solvent was added to the vial containing the aqueous layer and the mixture was stirred vigorously under N₂ atmosphere at room temperature for another 15 minutes. Again the top, aqueous layer was drawn off with a pipette and the remaining organic layer was combined with the first organic layer. The combined organic layers were dried over magnesium sulfate (MgSO₄) and filtered through a cotton-packed Pasteur pipette. The solvent from the resulting filtrate was evaporated from the solution under a stream of N₂ to yield 8 mg (+/- 4mg, n = 3).

2.6 Analytical Thin Layer Chromatography

The dried organic layer isolated from Folch extraction was analyzed by thin layer chromatography (TLC) using aluminum-backed, silica gel RP-18 plates (20cm × 20cm) treated with F_{254s} indicator (EM Science, Gibbstown, NJ) as stationary phase and CHCl₃:MeOH: acetic acid (ACOH): water (H₂O) in a ratio of 90:8:1:1 as mobile phase. [25,26] The samples were re-dissolved in Folch solvent (1 mg/95 μ l). Control and lipid

standards (20 μ l), were introduced to the plates using microcapillary pipettes. The plate was developed, removed from the developing chamber and the solvent front marked. Plates were air-dried and sprayed with a solution of phosphomolybdic acid (20% in ethanol) and gently heated to ~80°C over 5–15 minutes to detect lipid components. Retention factors (R_f) for standards and detected components from treated samples and controls were calculated.

2.7 Preparative Thin Layer Chromatography: Separation of Lipid Fractions

Lipids isolated in the Folch extraction were further separated by preparative thin layer chromatography (TLC) using glass-backed, silica gel RP-18 plates (20cm $\times$ 20cm) treated with F_{254s} indicator (EM Science, Gibbstown, NJ) as stationary phase and CHCl₃: MeOH: acetic acid (AcOH): water (H₂O) in a ratio of 90:8:1:1 as mobile phase [Powell, 1980]. The residue isolated from the Folch extraction was dissolved in Folch solvent (7 mg/200 μ l). The solution (150 μ l, ~5.25mg) was applied to the preparative TLC plate. A portion of the unfractionated sample (50 μ l, 1.75mg) was reserved for assessment of total extract activity on the febrile response. The plate was developed, removed from the developing chamber and the solvent front marked. Lipid-specific fractions were generated by scraping horizontal bands from the plates corresponding to the R_f ranges for each lipid class: fraction I: phospholipids, lysophospholipids, free fatty acids (R_f = 0.05–0.30), fraction II: prostanoids (R_f = 0.45–0.80) and fraction III: steroids and steroidal esters (R_f = 0.85–1.0). Each band (silica gel and embedded lipids) scraped from the plate was transferred to a glass vial. The silica gel embedded with the lipid was extracted with CHCl₃: MeOH, (2:1, (v/v), 3 $\times$ 5 ml) by adding extraction solvent to the vial and then vigorously stirring under N₂ for 10 minutes. The silica gel was removed by filtration and the filtrate transferred to a clean vial. Solvent was evaporated under a stream of N₂ to yield the lipid fraction (1–3mg).

2.8 Assessment of Extract Activity: Monitoring Febrile Response

Total isolated extracts (n=3) and lipid-specific fractions (I and II only, n=2) isolated by preparative TLC from LPS-treated guinea pigs and control animals were assessed for activity in limiting the febrile response to LPS. The dose of total extract (lipid mixture) was approximately 26mg/kg and the dose of each lipid fraction (I and II) was approximately 6mg/kg. To assess the inhibitory activity of the extracts on the febrile response stimulated by LPS, each isolated extract was injected into guinea pigs co-treated with LPS. The febrile response was monitored over 6 hours as described previously [Feleder et. al., 2005].

2.9 Solid Phase Extraction for HPLC and LCMS/MS Analysis of Spleen Extract

The splenic extract (2–3ml) was acidified with 1M HCl to pH 3.0 and immediately applied to Sep-Pak C₁₈ cartridges (1ml/each, 1ml cartridge, Waters Corp, Milford, MA) that had been pre-conditioned with methanol (2ml) and water (2ml) [Powell, 1980; Powell, 1982]. Cartridges were then washed with 10% methanol/water (2ml), and then the PGs were eluted with methyl formate (2ml). The extraction procedure was performed using a vacuum manifold, and the vacuum was adjusted so that individual drops could be seen from each cartridges. The organic solvent was evaporated under a stream of nitrogen; the residue containing PGs was reconstituted in 150 μ l of methanol, flushed with nitrogen and stored at –80°C, awaiting LC-MS/MS analysis [Salmon et al., 1982; Myher et. al, 1995]. Recovery of each PG from the extraction procedure was assessed using PG standards. Recoveries of 84%, 80%, 89%, 90% and 74% for 6-keto-PGF_{1 α} , PGF_{2 α} , PGE₂, PGD₂ and PGJ₂ respectively were determined.

2.10 HPLC Analysis of Total Spleen Extract

Extracts obtained after SPE were analyzed qualitatively and quantitatively for PG by reverse-phase (RP) high pressure liquid chromatography (HPLC) [Deems et. al., 2007; Kiss

et. al., 2008]. RP-HPLC was performed using a Waters 600 controller and solvent delivery system, a Waters 717 plus auto-sampler, and a Waters 996 photodiode array detector ($\lambda = 192\text{nm}$), which were interfaced directly with the mass spectrometer (Waters Acquity TQD tandem mass spectrometer (MS/MS). A RP-C₁₈ Gemini column (5 μ , 2.0 $\times$ 250 mm, Phenomenex, Torrance, CA) with an isocratic mobile phase (water:acetonitrile:formic acid, 58:42:0.01) at a flow rate of 250 μ l/min at 25°C were used for the HPLC analysis. Samples of spleen extract were dissolved in methanol and injection volumes of 20 μ l were used [Deems et. al., 2007]. HPLC analysis was done on five PG standards and the splenic samples. At a flow rate of 250 μ l/min at 25°C, excellent chromatographic separation among the PGs was achieved.

2.11 LC/MS/MS Analysis of Spleen Extract

Mass spectrometry was performed on a Waters Acquity TQD tandem mass spectrometer (MS/MS). The LC-MS/MS system was controlled by Waters Empower 2 Software package on a Windows based workstation. Optimization of the mass spectrometer was achieved using the Intellistart system (Waters), combined with manual refinements. Ionization was achieved using electrospray in the negative ESI mode. The position of the ESI probe and parameters of MS and MS/MS tuning were optimized for maximum sensitivity by introducing the prostaglandin standard solution (10ng/ μ l) infusion through a microsyringe pump (10 μ l/min) into the HPLC solvent flow (240 μ l/min). The mass-to-charge ratio (m/z) of the precursor ion of an individual compound was determined in the MS scan mode and the sample cone voltage for maximum signal intensity of the deprotonated species $[\text{M-H}]^-$ was determined in the selected-ion reaction (SIR) mode. Collision induced dissociation of each compound molecule was performed using MS/MS tuning. The most abundant product ion was determined in the product scan mode, and the collision energy for maximum signal intensity of precursor-product ions was determined in the multiple reaction monitoring (MRM) modes. Two of the strong and characteristic MRM scanning channels were selected to identify and confirm the PGs [Kiss et. al., 2008]. Nitrogen gas was used as the desolvation gas and argon was used as the collision gas. The optimized cone voltage and collision energy for each PG as shown in instrument method are also given in Table 1. An inter-channel delay time of 0.005s, inter-scan delay time of 0.005s, dwell time of 0.04s and span of 0.2 Da were used in data acquisition. To establish a standardized curve of concentration to peak intensity, a series of dilutions were made for all PGs. A linear regression curve was generated on the data comparing concentration to peak intensity.

Standard stock solutions were prepared in methanol (1mg/ml) and stored at -80°C . Composite standard solutions were prepared by mixing and diluting the appropriate stock solutions to the final concentration of 1.25pg/ μ l, 2.5pg/ μ l, 5pg/ μ l, 12.5pg/ μ l, 25pg/ μ l 50pg/ μ l, 125pg/ μ l and 250pg/ μ l. The injection volume was 20 μ l. The calibration curves were calculated by the least squares linear regression method. The limit of detection (LOD) was calculated by using a signal to noise (s/n) ratio of 3. The limit of quantification (LOQ) was determined by using a signal to noise (s/n) ratio of 10. Peak integration and s/n calculations were performed using waters Empower 2 software.

The recovery of PGs was determined at two different concentrations (25 and 125pg/ μ l) by comparing the peak areas of spiked and processed solutions (PBS, pH=3) with the corresponding standard solutions analyzed without extraction. The intra- and inter-day accuracy and precision for calibration samples were determined by estimating the corresponding response 5 times on the same day and on 5 different days over a period of one week for two different concentrations (5 and 50pg/ μ l). The accuracy was expressed as a percentage of nominal concentration. The % relative standard deviation (R.S.D) of the mean served as the measure of precision.

2.12 Data analysis

The PGs concentration in spleen is expressed as ng per g wet tissue. All the data were presented as mean $\pm$ S.E.M. Statistical analysis were evaluated using one-way analysis of variance (ANOVA) followed by Student's *t*-test. $P < 0.05$ were considered as statistically significant.

Results

3.1 Protein concentration in Spleen Extracts

Protein concentration assays were run in duplicate. The values are calculated according to the calibration curve (not shown). Protein concentrations in the splenic extracts are negligible based on the results of this assay.

3.2 Identification of Lipids by Analytical TLC

The results of the analytical TLC of the total splenic extract as compared with lipid standards are shown in Figure 1. Five discernable spots were detected on the TLC for the LPS-treated spleen extract (#11). Spots in this sample were detected with *R_f* values of 0.16, 0.48, 0.61, 0.86, 0.93. The *R_f* values of the components in the LPS-treated spleen extract correlate with three general classes of lipids based on comparison to *R_f* values for lipid standards. The spot at *R_f* = 0.16 correlates with polar lipids, phospholipid or lysophospholipid (fraction I). The spots at *R_f* = 0.48, 0.61, 0.86 correlate with non-polar lipid prostanoids PGE₂, PGF_{2 α} and TXB₂ (fraction II), and the spot at *R_f* = 0.93 correlates with very non-polar steroidal type lipids (fraction III). By comparison, the control sample contained spots with *R_f* values in roughly the same ranges as the LPS-treated spleen extract. However, based on visual inspection of the plate and the intensity of specific spots corresponding to the lipid components, the quantitative distribution of lipids in the LPS-treated spleen extract appears to be different from control.

3.3 Separation of lipid fractions by preparative TLC

Three fractions of lipids were collected. I: 1.0–1.2 mg; II: 0.8–1.0 mg; III: 0.9–1.3 mg. Separation of the lipids by preparative TLC removed any residual peptides and proteins [22–25]. The acidic silica gel that is used in this method retards peptides at the origin of the stationary phase, eliminating any peptides or proteins from fractions I and II [Salmon et. al., 1982; Skarnes et. al., 1981].

3.4 Assessment of Antipyretic Activity: Total Lipid Extract and Lipid Fractions

Isolation of the total lipid components from the spleen extract and analysis by TLC show the presence of lipids that strongly correlate with thromboxane B₂, prostaglandin F_{2 α} , cholesterol or cholesterol esters (steroidal lipids) and possibly phospholipids, lysophospholipids or sphingomyelin. LPS (8 μ g/kg, ip) induced a characteristic fever with an onset of latency of $\sim$ 70 min and peak magnitudes of 0.9°C occurring at 160 min, then gradually returned to basal T_c; recovery was essentially complete by the end of the experiment. The administration of the total lipid extract 10 min prior to LPS caused almost a complete inhibition of the febrile response ($P < 0.05$), with peak magnitudes of 0.3°C occurring at 120 min. Thereafter, T_c gradually returned toward basal levels. Therefore, total lipid extract (2mg/300g) induced a strong inhibition of the febrile response relative to sham (Figure 2). Separated fractions of specific lipid types did not affect the febrile response to LPS.

3.4 Quantitative HPLC and LC-MS/MS Analysis of PG Standards

A mixture of commercial prostaglandin standards (10ng/μl in MeOH) (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, PGD₂ and PGJ₂) was analyzed by HPLC. Our chromatogram for these PG standards is consistent with the chromatogram previously reported [Deems et. al., 2007]. LC-MS/MS conditions for analysis of PGs in spleen extracts were optimized based on the results from the HPLC analysis of the standards.

The sensitivity and linearity of quantification for each PG were determined. Standard curves were run using commercial PGs in a range from 25pg-5ng and were used to determine the concentrations of specific PGs in the spleen extract. Calibration curves showed excellent linearity with satisfactory correlation coefficients for the detected PGs ($R^2 > 0.998$). Correlation coefficients, LOD and LOQ data for each PG are shown in Table 2. Extraction efficiencies were also determined using the PG standards. Extraction recoveries at two different concentrations for each PG were determined and ranged from 75–94%.

The accuracy and precision of the PG measurements was also assessed. The intra-day precision ranged from 0.6 to 4.8% and the accuracy was 96.0 to 100.1%. The inter-day precision was 0.5 to 3.1% with an accuracy of 96.9 to 100.0%. The results for all analytes indicated that the LC/MS method showed great accuracy and precision.

3.6 Qualitative and Quantitative LC-MS/MS analysis of PG in spleen extracts

Four PGs were consistently found in both the LPS and saline-treated spleens as determined by our LC-MS/MS analysis. Concentrations of PGE₂ and PGD₂ (0.8–1.1pg/mg and 1.2–1.9pg/mg respectively) were consistently lower in both the LPS and control groups relative to 6-keto-PGF_{1α} and PGF_{2α}. No PGJ₂ was detected in either of the spleen samples. The levels of all four PGs were significantly higher in the LPS-treated spleens than in the saline controls (Figure 4).

Discussion

Previously, it was demonstrated that a protein-free splenic extract inhibits the exaggerated febrile response to LPS in guinea pigs [Feleder et. al., 2005]. The aim of the present study was to further identify and characterize PGs as potential bioactive factors in the splenic extract that contribute to this inhibitory effect. Our major goal was to develop a method to simultaneously qualitatively and quantitatively characterize prostaglandin profiles (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, PGD₂, and PGJ₂) in the splenic extract, since prostanoids have long been known to be essential regulators of the febrile response [Milton, 1998]. PGE₂ has previously been reported to be released by the spleen in response to LPS challenge, gram-negative bacteria, and during hemorrhagic and septic shock, and it is believed to be the principle mediator in the febrile response to LPS [Marcus, 1984]. It has long been known as both an immunoactivator and an immunosuppressant [Sakata et. al., 2010; Ushikubi et. al., 2000]. PGE₂ also plays an important role in febrile response, both in the neutral brain region or from the periphery, and at the initiation of fever and during the later phase [Skarnes et. al., 1981]. While PGF_{2α} also exhibits thermoregulatory activity, its potency is significantly less than that of PGE₂ [Milton, 1998]. The role of PGD₂ in fever is more complicated. Both hyperthermic and hypothermic effects have been observed after central administration of PGD₂ in rats [Gao et al., 2009; Ueno et. al., 1982] and more recently it has been reported that PGD₂ produced peripherally by H-PGDS contributes to LPS-induced fever [Gao et. al., 2009a]. 6-keto-PGF_{1α} is the stable metabolite of prostacyclin and PGJ₂ is the metabolite of PGD₂. PGJ₂ can be further metabolized to 15d-PGJ₂ which can cause hypothermia [Mouihate et. al., 2004]. Also, we have recently found that the intravenous administration of PGD₂ enhanced hypothermia in LPS treated guinea pigs (data not published).

Our first task was to harvest spleens from LPS-challenged guinea pigs and controls and isolate protein-free lipid fractions containing prostanoids. After homogenization, centrifugation and ultrafiltration, protein analysis was done to verify that the extracts were indeed protein-free. Two methods were then used to extract the targeted lipid components from the protein-free spleen homogenates. In the first approach, lipid components were extracted from the spleen using the classic Folch method [Folch et. al., 1957]. The Folch solvent used to extract lipid components is a non-polar medium that will predominantly solvate and extract lipids, leaving behind peptides containing ionizable residues. Results from the protein assay suggest that the total extract does not contain any detectable protein or small peptide. However the sensitivity of the method is limited and in fact very low concentrations of active proteins or peptide could still be present but not detectable in this extract. Further separation of the lipids by preparative TLC or SPE removed any residual peptides and proteins. We were able to isolate ~2–6 mg (~0.6% recovery) of lipid extract from each spleen.

Analysis by TLC of the total lipids in the spleen homogenates isolated by Folch extraction showed the presence of lipids that strongly correlate with prostanoids, cholesterol or cholesterol esters (steroidal lipids) and possibly phospholipids, lysophospholipids or sphingomyelin (Figure 1). Lipid profiles in both the LPS and control spleens were quite similar.

To validate that the lipids isolated from the Folch extraction elicit an antipyretic effect, extracts from donor guinea pig spleens (LPS and control) were co-injected with LPS (iv) into splenectomized (Splex) and sham animals. Co-injection of LPS with both the control and the LPS extracts (26mg/kg) resulted in modulation of the febrile response, with the LPS extract exhibiting a greater antipyretic effect (Figure 2). Thus, these data further support our hypothesis that the antipyretic factor in the spleen is indeed a lipid. We also attempted to isolate specific lipid fractions, utilizing preparative TLC. Two lipid-specific fractions were isolated (1–2 mg each) (I: $R_f = 0.05\text{--}0.30$: phospholipids, lysophospholipids, free fatty acids; II: ($R_f = 0.45\text{--}0.80$) eicosanoids) from the donor guinea pig spleens (control and LPS-treated). However neither fraction affected the febrile response to LPS. This failure to produce an antipyretic effect in fractions I and II may be due to the lower dose (6mg/kg versus 26mg/kg) administered to the guinea pigs.

Next, we focused on the qualitative and quantitative analysis of five major PGs (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, PGD₂, and PGJ₂) in the extracts by LC-MS/MS. Qualitative and quantitative analyses of prostanoids generated in tissues have been accomplished utilizing a variety of methods, including immunoassays (i.e. radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EIA)) [Dieter, 1994]. However, these immunoassays require highly specific and sensitive antibodies for each prostaglandin to be quantified, and they do not allow for the simultaneous identification and quantification of multiple PGs since many of the antibodies are cross-reactive with related compounds. The radioisotope method also requires specialized licensing, training, handling, and disposal of radioactive wastes. Gas chromatography-MS (GC-MS) methods have also been used to analyze PGs and this method greatly improves upon the limitations of immunoassays, but requires laborious sample preparation including derivatization [Wilson et. al, 1976]. HPLC analysis of PGs has also been reported and PGs can be easily separated by HPLC. However, the detection of PG is difficult because their UV-absorbance is ~200nm, and the sensitivity at this wavelength is inadequate [Henke et. al., 1984]. Therefore, to overcome the limitations of these other methods, we utilized an LC-electrospray ionization (ESI) tandem mass spectrometry (MS/MS) method to identify and quantify five PGs in a single run. The PGs carboxylate moiety readily ionizes in the ESI source and it allows the PGs to be analyzed by MS directly from an aqueous sample [Deems et. al., 2007; Yue et. al., 2007].

LC-MS/MS conditions were developed and optimized using five PG standards (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, PGD₂ and PGJ₂). Extraction efficiencies, linearity of response, limits of detection, and accuracy and precision of each run were determined, and taken into account when calculating the concentrations of PGs in the splenic extracts. Analysis of the splenic extracts showed both the control and LPS-treated samples contained four PGs (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, and PGD₂). No PGJ₂ was detected. The ratio among the four PGs in both groups was also similar (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, and PGD₂, 6.6:8.8:1:1.6) with 6-keto-PGF_{1α} and PGF_{2α} consistently having the highest concentrations. Splenic extracts derived from LPS-challenged donor guinea pigs showed significantly higher levels of all four PGs than the control group. To our knowledge, simultaneous qualitative and quantitative characterization of prostaglandin profiles in the guinea pig spleen has not been previously reported. The concentrations we have measured are consistent with PG concentrations in other mammalian tissues, including rat brain [Borja-Garcia-Bueno et al., 2008]. Our observation that PG levels become elevated under LPS challenge is also consistent with previous reports that show increases in PGE₂ levels in injured brain tissue.

The profile and ratio of the four PGs (6-keto-PGF_{1α}, PGF_{2α}, PGE₂, and PGD₂) are maintained when comparing the LPS group to the control group. We did not observe the appearance of a new PG as a result of LPS-challenge nor did we see a specific elevation or decrease in the concentration of one or more specific PG resulting in a change of the PG ratio. The only difference between the control and LPS-challenged group was a net increase in concentration of all four PGs detected. This observation does not definitively confirm that PGs are exclusively responsible for modulation of fever, only that PGs are present in the extract at an elevated concentration in response to LPS. Other factors in the extract may contribute to, or be solely responsible for the observed anti-pyretic effect. However, if PGs are involved in the antipyretic effect, such an effect is likely associated with an elevated concentration of a single ubiquitous PG or a combination of PGs PGE₂ and PGD₂ are the two prostanoids identified in the profile that are closely associated with thermoregulatory modulation. We recently found that PGD₂ injected systemically enhanced hypothermia in LPS treated guinea pigs (data not published). In contrast, PGE₂ failed to modify the hypothermic effect induced by LPS (data not published). These findings are consistent with our previous studies on food deprivation and thermoregulation [Krall et al., 2010].

Conclusion

In summary, we have utilized a combination of analytical and preparative chromatographic methods, coupled with mass spectrometry to isolate and characterize a profile of prostaglandins in the guinea pig spleen. We have applied this method to the characterization and comparison of prostaglandin profiles in control and LPS-challenged guinea pig spleens and have shown elevated levels of four prostaglandins in the LPS-challenged group. This method to measure PG profiles offers significant advantages over previously reported methods since it allows for simultaneous qualitative and quantitative analysis of PGs, and will be used in our future studies to investigate the role of prostaglandins in physiological processes associated with fever regulation.

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	Sample/Standard
1	Phosphatidylcholine
2	Phosphatidylethanolamine
3	Cholesterol
4	Cholesterol linoleate
5	Sphingomyelin
6	Thromboxane B ₂
7	Prostaglandin E ₂
8	Prostaglandin F _{2α}
9	L-α-Lysophosphatidylcholine
10	L-α-Lysophosphatidylethanolamine
11	LPS Extract
12	Control Extract
13	Folch Extraction Solvent
14	Mixture of 1-10

Figure 1.

Figure 2.

Figure 3.

Prostaglandins in Spleen Extract

Figure 4.

Table 1

Optimized MS tune parameters, MS/MS conditions and MRM ion fragmentation patterns for the prostaglandins.

Optimized MS Tune Parameters			
Source Parameters		Analyzer Parameters	
Capillary Voltage (kV)	3.60	LM1 Resolution (Da)	15.0
Extractor Voltage (V)	1	HM1 Resolution (Da)	15.0
RF Lens (V)	0.1	Ion Energy 1 (V)	0.6
Source Temperature (°C)	150	MS Mode Entrance (V)	50.00
Desolvation Temperature (°C)	400	MS Mode Collision Energy (eV)	2.00
Cone Gas Flow (L/hr)	50	MS Mode Exit (V)	50.00
Desolvation Gas Flow (L/hr)	900	LM2 Resolution (Da)	13.0
Collision Gas Flow (ml/min)	0.28	HM2 Resolution (Da)	13.0
		Ion Energy 2 (V)	2.0
		MSMS Mode Entrance (V)	-5.00
		MSMS Mode Exit (V)	5.00
		Gain	1.00
MS/MS conditions and MRM ion fragmentation patterns			
	Precursor → Product	Cone Voltage(V)	Collision Voltage (eV)
6-keto-PGF _{1α}	369.2 → 245	40	26
	369.2 → 163	40	26
PGF _{2α}	353.2 → 309	38	18
	353.2 → 193	38	24
PGE ₂	351.2 → 271	25	16
	351.2 → 189	25	20
PGD ₂	351.2 → 271	25	16
	351.2 → 189	25	20
PGJ ₂	333.2 → 271	21	16
	333.2 → 189	21	18

Table 2

Sensitivity, linearity and percent recovery of prostaglandins

Compound	6-keto- PGF _{1α}	PGF _{2α}	PGE ₂	PGD ₂	PGJ ₂
<i>Sensitivity and Linearity</i>					
LOD (pg)	5.1	11.6	8.0	18.1	40.2
LOQ (pg)	15.3	34.8	24.0	54.3	120.6
Line Equation	$y = 639x + 36500$	$y = 496x + 33300$	$y = 568x + 34600$	$y = 399x + 20400$	$y = 329x + 21200$
R ²	0.999	0.999	0.998	0.998	0.998
<i>Percent Recovery</i>					
Concentration (pg)/Recovery (%)	500/85.9 ± 3.4	500/90.0 ± 3.7	500/91.4 ± 3.4	500/84.9 ± 3.9	500/76.1 ± 3.8
	2500/89.5 ± 4.4	2500/90.0 ± 1.8	2500/94.0 ± 2.8	2500/87.6 ± 4.3	2500/75.0 ± 2.8