Determination of isoprostane 8-iso-PGF2α and prostaglandin GF2α in plasma and intestine of specific-pathogen-free chickens challenged with Eimeria maxima

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Research Article

Abstract

The purpose of this study was to evaluate and determine the concentration of isoprostane 8-iso-PGF2α and prostaglandin GF2α (PGF2α) from plasma and intestine in specific pathogen-free (SPF) chickens challenged with Eimeria maxima (EM) using solid-phase microextraction and ultra-performance liquid chromatography/tandem mass spectrometry. Forty one-day-old male SPF chickens were randomly allocated to one of two groups with four replicates (n=5 chickens/replicate). Groups consisted of Control (no challenge) or the Challenge group EM (40,000 sporulated oocysts/bird). At day 7 and 9 post-challenge, half of the chickens were euthanized in both groups to determine plasmatic and enteric concentrations of isoprostane 8-iso-PGF2α and PGF2α. Enteric levels of both 8-iso-PGF2α and PGF2α were significantly increased at 7 (8-iso-PGF2α P=0.0000252; PGF2α P=0.00000268) and 9 days (8-iso-PGF2α P=0.000000717; PGF2α P=0.00000222) post-challenge compared to non-challenge control chickens. However, plasma levels of isoprostane 8-iso-PGF2α and PGF2α were similar in both groups. A significant reduction (P=0.0000095) in oocyst excretion was observed in chickens at 9 days post-challenge compared to 7 days. Chickens challenged with EM showed an inflammatory response associated with significant increases in enteric PGF2α and 8-Iso-PGF2α, suggesting that the active disease phase was accompanied by inflammation and oxidative stress within the intestinal layer.

Introduction

Coccidiosis is a parasitic enteric disease of animals caused by coccidian protozoa from the Apicomplexa phylum. In a recent study, the global cost of coccidiosis in broiler chickens was estimated at ~£10.36 billion[1]. *Eimeria* spp. have a remarkable and complex life cycle, including sexual and asexual reproduction with intracellular and extracellular phases[2,3,4]. Hence, during the disease, the gut-associated lymphoid tissues respond with a series of innate and acquired immune reactions against the parasite[5,6]. Several investigators have extensively studied and documented the immunopathology of cellular responses involving the secretion of pro-inflammatory cytokines to *Eimeria* infections in chickens[7,8,9,10,11,12]. However, little is known about the role of prostaglandins (PG) and isoprostanes (F$_2$-Ips) as part of the innate response during clinical coccidiosis. Prostaglandins are a group of lipid compounds from the eicosanoid family implicated in inflammation, allergy, fever, and other immune responses that are generated from arachidonic acid by the action of cyclooxygenases (COXs) isoenzymes. On the other hand, F$_2$-Ips are PG-like complexes formed from free radical catalyzed oxidation of arachidonic acid, without the action of COXs. The measurement of F$_2$-Ips, especially 8-epi-PGF$_{2α}$, is recognized as a consistent biomarker of lipid peroxidation and is currently used as a sensitive index of oxidative stress *in vivo*.

The purpose of this study was to evaluate and determine the concentration of prostaglandin GF2α (PGF2α) and isoprostane 8-iso-PGF2α in plasma and intestine of specific pathogen-free (SPF) chickens challenged with *Eimeria maxima* using solid-phase microextraction and ultra-performance liquid chromatography/tandem mass spectrometry.

Results

The evaluation of isoprostane 8-iso-PGF2α and PGF2α from jejunum and plasma in SPF chickens challenged with *Eimeria maxima* at 7- and 9-days post-challenge are summarized in Table 1. Enteric levels of both isoprostane 8-iso-PGF2α (7 days: P=0.0000252; 9 days: P=0.000000717) and PGF2α (7 days: P=0.00000268; 9 days: P=0. 00000222) were significantly increased (P < 0.01) at both time points compared to the non-challenge control chickens (Table 1; Figure 2). However, plasma levels of isoprostane 8-iso-PGF2α (7 days: P=0.29812; 9 days: P=0.2923) and PGF2α (7 days: P=0.021489; 9 days: P=0.0403) were similar (P>0.01) in both groups (Table 1; Figure 3). Table 2 shows the results of *E. maxima* OPG of feces from the SPF chickens at 7- and 9-days post-challenge. As expected, challenged chickens showed a significant increase (7 days: P= 0.00000095; 9 days: P= 0.00000095) in oocyst excretion when compared with non-challenge control chickens. Moreover, a significant reduction (P<0.05) in oocyst excretion was observed in chickens at 9 days post-challenge compared with day 7 (P= 0.00002) (Table 2).

Discussion

In addition to the critical job of absorbing water and nutrients, enterocytes also play an essential role in the mucosal immune response, maintaining tolerance to beneficial microbiota, and identifying luminal pathogens. The invasion of *Eimeria* spp in intestinal epithelial cells is a complex process that includes several events beginning with the excystation of sporozoites after oral ingestion of the oocysts[13,14]. As intracellular parasites, attachment and invasion of the sporozoites to the host cell is recognized by Toll-like receptors 4 and 15, involved in pathogen recognition and activation of the mucosal inflammasome IL-1/IL-18 axis, which is responsible for recruiting and activating heterophils, natural killer cells, mast cells, macrophages, and the transcription factor NF-κB[15,16,17,18]. Nevertheless, sporozoites have evolved a unique molecular system fueling motility and invasion of epithelial cells through gliding motility, allowing them to rapidly invade host cells and form an intracellular parasitophorous vacuole that protects them from the intracellular hostile environment[19,20,21,22]. Within this vacuole, these Apicomplexa parasites gain precious time to continue with their multifaceted life cycle. Each phase of the sexual, asexual, intracellular, or extracellular stages of this prehistoric and remarkable parasite are associated with severe local inflammation, autophagy, apoptosis, cellular death, hemorrhages, and necrosis in the intestinal mucosa[20,21,23,24,25]. Hence, coccidia infections are characteristic by inducing chronic inflammation and excessive tissue damage because of the parasite infection and the host immune response elicited against the invaders. In chickens, macrophages are the primary sources of nitric oxide, superoxide, and hydrogen peroxide as essential mediators of both innate and acquired immunity, thus increasing during coccidia infections[26,27,28,29,30]. In the present study, chickens challenged with *E. maxima* showed a significant increase (P <0.01) in enteric PGF2α at 7- (P=0.00000268) and 9-days (P=0.00000222) post-challenge when compared with non-challenge chickens. However, the serum levels of PGF2α remained similar in both groups (Table 1). PGs are produced from arachidonic acid release from phospholipids in the cellular membrane by cyclooxygenases (COXs). They are fundamental in generating inflammatory

responses against pathogens[31,32]. While they have a quick action during the acute phases of the inflammatory response, there is crosstalk with cytokines to synergistically activate NF-κB factor and induce gene expression of pro-inflammatory cytokines and more COXs, mediating positive feedback loops and consequently, chronic inflammation[33,34].

Since the cellular components that suffer immediate damage are the lipids and proteins of the cell membrane and mitochondrial membrane by lipid peroxidation, the whole-cell physiology is then compromised. One of the cellular mechanisms to revert oxidative stress is the production of several heat shock proteins that repair damage proteins and regulate apoptosis[35,36,37]. A noteworthy result observed in this study was the significant increase ($P<0.01$) in isoprostane 8-iso-PGF2α in the jejunum of chickens challenged with *E. maxima* at 7- (P=0.0000252) and 9-days (P=0.000000717) post-challenge compared to the non-challenge control chickens. Excessive generation of reactive oxygen species has been implicated in a variety of pathological events. However, lipid peroxidation is the primary marker of oxidative stress in many pathological conditions, so isoprostanes are reliable biomarkers evaluate it[38,39]. In contrast, F2-isoprostanes (8-Iso-PGF2α) have harmful and potent bioactivities, including vasoconstriction, platelet aggregation, and cardiac hypertrophy[40,41,42,43]. As far as we know, this is the first report of detection of 8-Iso-PGF2α following a challenge of *E. maxima* in the jejunum (Table 1), despite plasma levels of 8-Iso-PGF2α remaining similar in both groups. It is known that in humans, the plasma half-life of 8-Iso-PGF2α is one minute at the distribution stage and the removal stage half-life is four minutes[44]. Hence, the half-life in chicken plasma may also be short, which may be why we were not able to detect it. However, pharmacokinetic and metabolic studies evaluating earlier points as well as daily oocyst count are required to confirm and extend these results.

In summary, in the present study, SPF chickens challenged with *E. maxima* showed an inflammatory response associated with a significant increase (7 days: P=0.00000268; 9 days: P=0.00000222) in enteric PGF2α. These changes were related to a significant increase (7 days: P=0.0000252; 9 days: P=0.000000717) of enteric 8-Iso-PGF2α and oocyst excretion, suggesting that the active disease phase was accompanied by inflammation and oxidative stress within the intestinal layer. Since polyunsaturated fatty acids and cholesterol are the principal targets of oxidative stress, lipid peroxidation end products such as 8-Iso-PGF2α are also part of the pathogenesis of inflammation-related changes caused by *E. maxima*, confirming the role of 8-Iso-PGF2α as a sensitive biomarker of oxidative stress in chickens. Further studies to control oxidative damage and subsequently intestinal mucosal over-production of lipid oxidation products using phytobiotics, such as essential oils in the diet with specific antioxidants anti-inflammatory and coccidicidal properties, are currently being evaluated.

Methods

Challenge strain

Eimeria maxima M6 oocysts were provided by Dr. John. R. Barta, University of Guelph, Canada. The methods for detecting and recovering oocysts from challenged chickens, oocyst sporulation, and the

preparation of infective doses were conducted as described previously[45,46].

Animal source and experimental design

Forty one-day-old male SPF chickens (ALPES® Tehuacan, Puebla, Mexico) were randomly allocated to one of two groups with four replicates per group (n=5 chickens/replicate). Chickens were placed in battery cages with a controlled age-appropriate environment at the diagnostic laboratory of the Avian Medicine Department of the Faculty of Veterinary Medicine and Zootechnics (FMVZ) at the National Autonomous University of Mexico (UNAM). Groups consisted of the Control (no challenge) or the Challenge group (*E. maxima*). Chickens were provided with *ad libitum* access to water and unmedicated feed. At day 28 of age, all chickens in the challenge group were orally gavaged with 40,000 sporulated *E. maxima* oocysts in a volume of 1 mL of sterile phosphate-buffered saline solution (PBS). The dose was selected based on a previous trial conducted to determine a challenge dose causing sub-clinical coccidiosis as described previously[13]. Negative control chickens were sham inoculated with 1 mL of PBS. Seven days after challenge, all chickens were bled, and half of them were euthanized to collect the second half of the jejunum to determine plasma and enteric concentrations of isoprostane 8-iso-PGF2α and PGF2α. At 9 days post-challenge, the rest of the chickens in both groups were bled and jejunum was collected to perform the evaluations. Oocysts per gram (OPG) of feces were evaluated at 7- and 9-days post-challenge.

The standards for 8-iso-PGF2α and 8-iso-PGF2α-d4

The standards for 8-iso-PGF2α and 8-iso-PGF2α-d4 (internal standard) were purchased from Cayman Chemicals (Ann Arbor, MI), while the standard for PGF2α was obtained from Sigma-Aldrich (St Louis, MO). Acetonitrile and methanol (HPLC grade) were purchased from JT Baker. Milli-Q water (Millipore system) was used throughout the experiments. Formic acid (FA: 95%, reactive grade) and isopropanol (LC/MS grade) were purchased from Sigma-Aldrich (St Louis, MO). Ammonium hydroxide (NH_4OH, reactive grade, 29.60%) and potassium hydroxide (KOH) were purchased from JT Baker. For solid-phase microextraction (micro-SPE), 96-well Oasis® MAX µElution cartridges containing a water-wettable reversed-phase strong ammonium exchange mixed-mode polymer, which is selective for acids and stable in organic eluents, were used. A Positive Pressure-96 processor purchased from Waters was also used. Figure 1 shows the chromatograms of standards.

Procedure for the extraction of 8-iso-PGF2α and PGF2α in chicken plasma

Extraction of 8-iso-PGF2α and PGF2α were determined as previously described[47]. An aliquot of 500 µL chicken plasma was transferred to 2 mL vials, followed by the addition of 100 µL of 4 ng/mL 8-iso-PGF2α-d4 as an internal standard and 500 µL of hydrolysis solution (KOH, 15%) to release 8-iso-PGF2α-esterified. The vials were mixed and incubated in an ultrasonic bath for 30 min at 40 °C. Subsequently, the vials were cooled to room temperature and 225 µL of 6M formic acid (FA) was added, mixed, and centrifuged at 15000 rpm for 10min at 4 °C. Solid-phase microextraction using a 96-well Oasis® MAX

µElution plate conditioned with 500 µL of methanol and 500 µL of 20 mM FA was used. Finally, the cartridges were loaded with 350 µL of plasma and washed with 350 µL of 2% NH$_4$OH. Samples were then eluted with 50 µL of a mixture of 5% FA in acetonitrile and isopropanol (40:60) and diluted with 150 µL of Milli-Q water. Samples were analyzed (30 µL) using ultra-performance liquid chromatography/tandem mass spectrometry (UPLC/MS/MS).

Procedure for the extraction of 8-iso-PGF2α and PGF2α in chicken intestine

For the extraction of 8-iso-PGF2α and PGF2α, 0.1 g of homogenized second half of the jejunum (Meckel's diverticulum to cecal tonsils) were transferred to 2 mL vials, followed by the addition of 100 µL of 4 ng/mL 8-iso-PGF2α-d4 as the internal standard and 1.5mL of chloroform: methanol (80:20) mixture. The vials were mixed 30s by vortex and 15 min in an ultrasonic bath. Samples were then centrifuged at 15000 rpm for 20 min. The supernatant was evaporated and 500 µL of methanol and 500 µL of hydrolysis solution (KOH 15%) were added, mixed, and incubated in an ultrasonic bath for 30 min at 40 °C. Subsequently, the vials were cooled to room temperature and 225 µL of 6 M formic acid (FA) and 50 µL of 88% FA were added, mixed, and centrifuged at 15000 rpm for 10min at 4 ºC. Solid-phase microextraction and analysis of samples were performed in the same way as for the determination of 8-iso-PGF2α and PGF2α in chicken plasma using a 96-well Oasis® MAX µElution plate conditioned with 500 µL of methanol and 500 µL of 20 mM FA. Finally, the cartridges were loaded with 350 µL of jejunum sample and washed with 350 µL of 2% NH$_4$OH. Samples were then eluted with 50 µL of a mixture of 5% FA in acetonitrile and isopropanol (40:60) and diluted with 150 µL of Milli-Q water. The sample (30 µL) was injected into a UPLC-MS/MS system for analysis, under the chromatographic and mass spectrometric conditions described previously by Rodriguez Patiño *et al*[47].

Ethics

This study was carried out in accordance with the recommendations for the management of chickens as recommended by the Internal Committee for Care and Use of Experimental Animals (CICUAE, from its abbreviation in Spanish) of the National Autonomous University of Mexico (UNAM), Ethical approval code CICUAE: C20_06, and the study is in compliance with the ARRIVE guidelines where animals are involved.

Quantification of oocysts

The quantification of OPG from feces was performed at 7- and 9-days post-challenge by using the McMaster technique as previously described[45].

Data and statistical analysis

PGF2α and 8-iso-PGF2α data are presented as means with standard deviation (S.D.). The number of samples per variable group was 20, implying a normal distribution (Shapiro-Wilk test), and the homoscedasticity was verified (Levene's test). Accordingly, the parametric test of one-tailed Student's t-

test was performed, and the P value was established with an alpha level of $P<0.01$. OPG data are presented as means with median and variance. The number of samples per variable group was 20; however, the hypotheses of normal distribution (Shapiro-Wilk test) and homoscedasticity (Levene's test) were not confirmed. Consequently, non-parametric tests of the one-tailed Wilcoxon signed-rank test was applied with an alpha level $P<0.05$[48].

Declarations

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Author Contributions

Conceptualization: VMP-G, RL-A, and GT-I. Investigation and Methodology: GRP, MACR, DH-P, BS-C, and XH-V. Formal analysis and Software: FA-H, CNV, IC-H. Supervision: VMP-G, and GT-I. Validation and Visualization: DH-P and BS-C. Writing-original draft: VP-G and GT-I. Writing-review and editing: XH-V and GT-I. All the authors reviewed, edited, and approved the manuscript.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Tables

Table 1. Evaluation of isoprostane 8-iso-PGF2α and prostaglandin GF2α from enteric (jejunum) and plasma of SPF chickens challenged with *Eimeria maxima* at 7 and 9 days post-challenge.

| | Prostaglandin GF2α | | Isoprostane 8-iso-PGF2α | |
	Enteric (pg/g)	Plasma (pg/mL)	Enteric (pg/g)	Plasma (pg/mL)
7 days post-challenge				
Control	6,934.47 ± 2497.11 [a]	107.92 ± 50.75	760.10 ± 329.39 [a]	97.17 ± 40.08
E. maxima	12,076.52 ± 3,445.58 [b]	151.50 ± 71.50	1,272.80 ± 366.60 [b]	101.99 ± 32.89
P value	*P*=0.00000268	*P*=0.021489	*P*=0.0000252	*P*=0.29812
9 days post-challenge				
Control	8,984.66 ± 2,629.54 [a]	162.05 ± 66.50	669.16 ± 355.16 [a]	110.55 ± 40.23
E. maxima	14,191.48 ± 3,356.85 [b]	124.38 ± 52.90	1,363.84 ± 398.59 [b]	105.33 ± 39.50
P value	*P*=0.00000222	*P*=0.0403	*P*=0.000000717	*P*=0.2923

Data expressed as mean ± standard deviation. n=20.

[ab] Different superscripts within columns and days indicate a significant difference at *P* < 0.01.

Table 2. *Eimeria maxima* oocyst per gram in the feces of SPF chickens at 7 and 9 days post-challenge.

	7 days post-challenge	9 days post-challenge	[y,z] *P* value
Control	0 ± 0 [a.y]	0 ± 0 [a.y]	*P*=0.50
E. maxima	24,240 (20,212.5; 0.37868) [b, y]	2,750 (1,700,0.823027) [b, z]	*P*=0.00002
[a,b] *P* value	*P*= 0.00000095	*P*= 0.00000095	

Each value represents the mean (median; variance). n=20.

[a-b] Values within groups columns, or [y-z] values within time of evaluation within columns with different superscripts differ significantly at *P* < 0.05.