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Expression and localization of cyclooxygenases in the oviduct of laying hens during the ovulatory cycle

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ABSTRACT

Prostaglandins (PGs) play important roles in regulation of the functions of the hen oviduct. However, little is known about the expression and localization of the rate-limiting cyclooxygenases (COX-1 and COX-2) in the oviduct. The aim of this study was to determine the COXs expression and localization in the different segments of the oviduct and to investigate changes in their expression levels during the ovulatory cycle of laying hens. White Leghorn laying hens were killed at 0, 4, 7, 16 and 24 h after oviposition, and samples from the infundibulum, magnum, isthmus, uterus, and vagina were collected. Gene and protein expressions were examined by real-time PCR and western blot, respectively, for both COX-1 and COX-2. Localization of COX-1 and COX-2 in the hen oviduct was determined by immunohistochemistry and PCR analysis of samples collected by laser capture microdissection (LCM). The expression level of *COX-1* was highest in the infundibulum, while that of *COX-2* was significantly higher in the uterus than in the other segments. The expression levels of *COX-1* in the infundibulum and *COX-2* in the uterus were higher at 0 and 24 h after oviposition, just prior to subsequent ovulation and oviposition. Western blot analysis confirmed the presence of COX-1 and COX-2 in all oviductal segments. The density of COX-2 was the highest in the uterus, and did not change during the ovulatory cycle. COX-1 and COX-2 were localized in the surface epithelium of all oviductal segments besides the uterine tubular glands. We conclude that both COXs are differentially expressed in the different oviductal segments with a temporal association to ovulation and oviposition. COX-1 and COX-2 may play an important role in the infundibulum and uterus, respectively, and COX-2 may be one of the factors regulating the induction of oviposition.

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1. Introduction

Prostaglandins (PGs) are one of the primary factors responsible for regulation of the functions of the hen oviduct, such as sperm storage in sperm storage tubules [1], motility of the oviduct during transportation of the ovum [2], eggshell calcification [3,4], and oviposition [5–9]. Synthesis of PGs in the oviduct of the laying hen was reported previously [4,10], and maintenance of PGs within physiological levels is important for normal egg formation [11]. PGs are supposed to act locally because they interact with their specific receptors, while released PGs in the serum are rapidly metabolized [12,13].

Cyclooxygenases (COXs; prostaglandin G/H synthase) are primary rate-limiting enzymes for the synthesis of PGs and thromboxanes. Two isoenzymes for COXs have been identified, namely the constitutive COX-1 and the inducible COX-2, which are encoded by the conserved *PTGS1* and *PTGS2* genes, respectively. COXs are responsible for converting arachidonic acid to PGH2, which is a substrate for production of different PGs [14,15]. COX-1 and -2 are expressed in the ovary of hens [16], quail [17], and ostrich [18], but little is known about their expression or localization in the hen oviduct. It is necessary to characterize the COXs expression profiles for better understanding the mechanism by which oviductal functions are regulated by PGs. The objective of this study was to characterize the expression profiles and localization of COX-1 and COX-2 in the oviduct of laying hens at different times during the ovulatory cycle. Specific questions included (1) which tissues expressed the COXs, (2) whether COX expression levels differ among the different oviductal segments, and (3) whether the

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expression levels change during an ovulatory cycle in each segment.

2. Materials and methods

2.1. Birds and tissue collection

Twenty-five White Leghorn laying hens (46–52 weeks old), regularly laying five or more eggs in a sequence, were kept in individual cages under a regimen of 14 h light: 10 h dark and provided with commercial feed and water *ad libitum*. Hens were killed at 0, 4, 7, 16, or 24 h after oviposition under anesthesia with sodium pentobarbital (Somnopentyl; Kyoritsu Shoji Co., Tokyo, Japan). Samples from different segments of oviduct including the infundibulum, magnum, isthmus, uterus, and vagina were collected with confirmation of egg location in the oviduct. The egg was absent at 0 h, in the isthmus at 4 h, and in the uterus at 7, 16, and 24 h after oviposition, whereas the last three times represented the initial, middle and final stages of eggshell formation. The handling of birds was performed in accordance with guidelines of the Animal Experiment Committee of Hiroshima University.

2.2. PCR analysis

2.2.1. RNA extraction

Tissue samples for extraction of total RNA from the whole mucosa were collected from the five different segments of the oviduct at five different time points during the ovulatory cycle. Extraction of RNA was performed using Sepasol-RNA I Super (Nacalai Tesque Inc., Kyoto, Japan) according to the manufacturer's instructions. Pelleted RNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and kept at −80 °C until use.

For extraction of total RNA using laser capture microdissection (LCM), uterine tissues (n = 4) at 0 h after oviposition were frozen in isopentane cooled with dry ice. Frozen sections (10 μm in thickness) were air-dried and fixed with acetone at −20 °C for 30 s, washed with ultrapure water (30 s × two times), and then gradually dehydrated using 70% and 95% (v/v) ethanol (30 s each) and 100% ethanol (90 s × two times) on ice. Subsequently, they were kept in xylene for 5 min at room temperature and air-dried. The cells of surface epithelium and uterine tubular glands were then collected separately, with approximately 200 spots for one sample, using an LCM system (LM200; Olympus, Tokyo, Japan). Total RNA extraction from the collected samples was performed using an RNAGEM Kit (ZyGEM Corp. Ltd., Hamilton, New Zealand) according to the manufacturer's instructions.

2.2.2. Reverse transcription (RT)

The RNA samples of the mucosal tissues were treated with RQ1 RNase-free DNase (Promega Co., Madison, WI, USA) on a programmable thermal controller (PTC-100; MJ Research, Waltham, MA, USA) at 37 °C for 45 min and then at 65 °C for 10 min. Concentrations of RNA from mucosal tissue were measured using Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, UK). Then RNA samples were reverse transcribed using ReverTra Ace (Toyobo Co. LTD., Osaka, Japan) according to the manufacturer's instructions. The RT reaction mixture (10 μL) consisted of 1 μg of total RNA, 1 × RT buffer, 1 mM deoxynucleotide triphosphate mixture, 20 U of RNase inhibitor, 0.5 μg of oligo (dT) 20, and 50 U ReverTra Ace. The RT was performed at 42 °C for 30 min, followed by heat inactivation for 5 min at 99 °C using the programmable thermal controller (PTC-100; MJ Research).

The RNA samples collected by LCM were also treated with RQ1 RNase-free DNase (Promega Co.) as mentioned above. The RT was performed with same conditions as above using the programmable thermal controller (PTC-100; MJ Research).

2.2.3. Polymerase chain reaction (PCR)

The PCR was performed using Takara Ex Taq Kit (Takara Bio. Inc., Shiga, Japan) on a thermal controller (PTC-100; MJ Research). Primer sequences of *COX-1* (XM_425326) were forward = TCAGGTGGTTCTGGGACATCA and reverse = TGTAGCCGTACTGGGAGTTGAA; *COX-2* (NM_001167719) primers were forward = CTGCTCCCTCCCATGTCAGA and reverse = CACGTGAAGAATTCCGGTGTT; and *RPS-17* (NM_204217.1) primers were forward = AAGCTGCAGGAGGAGGAGAGG and reverse = GGTTGGACAGGCTGCCGAAGT. The reaction mixture (25 μL) consisted of 0.5 μL of cDNA, 1 × Ex Taq buffer, 0.2 mM of deoxynucleotide triphosphate mixture, 0.625 U of Takara Ex Taq, and 0.4 μM of each primer. PCR products were electrophoretically separated on a 2% (w/v) agarose gel with 0.6 μg/mL ethidium bromide and examined on a transilluminator (NTM-10E; UVP LLC, Upland, CA, USA).

2.2.4. Quantitative real-time PCR (qRT-PCR)

The expression levels of target genes within each oviductal segment at different times of an ovulatory cycle were analyzed by real-time PCR using a Roche Light Cycler Nano System (Roche Applied Science, Indianapolis, IN, USA). The reaction mixture (10 μL) consisted of 0.5 μL cDNA, 1 × Thunderbird SYBR qPCR Mix (Toyobo Co. LTD., Osaka, Japan) and 250 nM of each primer. Amplification conditions for *COX-1* and *COX-2* were 50 °C for 2 min, 95 °C for 2 min, 50 cycles at 95 °C for 10 s, and 60 °C for 40 s. Amplification conditions *RPS-17* were 45 cycles at 95 °C for 30 s and 62 °C for 30 s.

Real-time PCR data were analyzed by the $2^{-\Delta\Delta CT}$ method to calculate the relative level of expression in each sample using the RPS-17 expression as the internal control. The results were shown as the expression level relative to the standard sample (a sample from the magnum at 4 h after oviposition). The expression levels were compared among the five oviductal segments at each of the five time points of ovulatory cycle, and among the five different time points of an ovulatory cycle within each of the five oviductal segments.

2.3. Immunohistochemistry

Samples from the middle part of each oviductal segment collected at 24 h after oviposition were fixed in 10% (v/v) formalin in phosphate buffered saline (PBS) (n = 4). They were processed for paraffin sections (4 μm in thickness). Immunostaining for COX-1 and COX-2 was performed using monoclonal antibody to human COX-1 (Abcam Inc., Cambridge MA, UK), and polyclonal antibody to human COX-2 (Cayman Chemical, Ann Arbor, MI, USA), which have been used to identify COX-1 and COX-2 in the hen ovary by Eilati et al. [19] and Hales at al. [16], respectively. Antigen retrieval was performed by heating at 98 °C in 10 mM trisodium citrate, pH 6.0, with 0.05% (v/v) Tween-20 for 30 and 10 min for COX-1 and COX-2, respectively. Endogenous peroxidases were quenched using 0.3% (v/v) H_2O_2 in methanol for 15 min. Endogenous biotin was blocked using Avidin/Biotin Blocking kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with 1% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich Japan K.K., Tokyo, Japan) for 1 h, followed by monoclonal anti-COX-1 or polyclonal anti-COX-2 antibodies overnight at 4 °C. The COX-1 and COX-2 antibodies were diluted in PBS, which contained 1% (w/v) BSA and 0.1% (v/v) tween-20. The final concentrations of the COX-1 and COX-2 antibody solutions were 0.15 and 1 μg/mL, respectively. Normal rabbit IgG (Santa Cruz Biotechnology, Inc. Texas, USA) diluted in the same way used for negative control staining. After washing by Tris-buffered

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