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Chicken Cyclo-oxygenases Response in a Pulmonary Infection Disease

Aida Karray*, Sofiane Bezzine

National School of Engineers of Sfax ENIS, University of Sfax, Sfax, Tunisia

Email address:
karrayaida_biotech@yahoo.fr (A. Karray)

*Corresponding author

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Abstract: Avian infectious bronchitis, associated coronavirus causes inflammation and damage to the lungs. To evaluate the molecular mechanisms behind this event, we investigated variations in the expression level of the proinflammatory factors, cyclo-oxygenase 1 (COX-1) and cyclo-oxygenases 2 (COX-2). In mammals, the increased generation of prostaglandins during the onset of inflammatory responses and activation of immune cell types has been attributed to the induction of COX-2. The recent sequencing of chicken genome and the identification of genes coding COX-1 and COX-2 allowed us to analyze their expression in lung inflammatory disease. The purpose of this study was to examine the relative expression and distribution of COX-1 and COX-2 in organs of normal hens and in hens with avian infection bronchitis. The expression levels of cyclo-oxygenase genes in several organs from uninfected chickens and infected ones were measured using a quantitative reverse transcription-PCR technique. Our results show that the expression level of COX-1 was highly up regulated in spleen, small intestine, lungs, and liver, but modestly up regulated in the pancreas. However, no significant change was observed in the expression levels of COX-2, in the small intestine, liver and spleen; suggesting a constitutive expression of this enzyme. Whereas, COX-2 mRNA expression was significantly decreased in the heart and lungs of infected chickens, ranging from 9-fold to 4-fold, respectively ($p<0.05$). Contrary to mammals, COX-2 is present constitutively in bird's cells, while COX-1 is expressed primarily after inflammation.

Keywords: Cyclo-oxygenase, Gene Expression, qPCR, *Gallus gallus*, Avian Infectious Bronchitis

1. Introduction

Chicken Infectious bronchitis (IB) is caused by a *coronavirus* belonging to the family of coronaviridae, genus Coronavirus. This type of virus is subdivided into three groups: group 1, group 2 and group 3 infecting human, bovine and birds, respectively. The coronavirus infecting humans is responsible for severe respiratory acute syndrome, [1] characterised by hypoxemia and non cardiogenic pulmonary oedema [2]. It is associated with lung surfactant disorders which can be observed soon after the initial injurious event and leads to increased surface tension, alveolar collapse, and deficiency of immune host defence [3, 4]. The group 3 coronavirus belonging to the same virus family includes only avian is the etiologic agent in infectious bronchitis virus (IBV) which is an acute characterized by tracheal rales, coughing, and sneezing highly contagious viral disease of the respiratory tract in chickens [5]. The disease development causes diarrhea, morbidity, and mortality, and the impact of coronavirose on the industry has serious economic consequences. Although effective vaccines are available and used routinely in commercial poultry production, the virus has the tendency to mutate frequently [6].

Cyclo-oxygenase (COX) is the rate-limiting enzyme in the biosynthesis of prostaglandins. It catalyzes the conversion of arachidonic acid into prostaglandin H2, the precursor for all prostaglandins, prostacyclins and, thromboxanes. Cyclo-oxygenase metabolites have a wide variety of physiological and pathophysiological effects and are involved in a number of homeostatic processes. However, in cardiovascular homeostasis, inflammatory oedema and pain COX have been considered as a therapeutic target. In mammals, two COX isoforms have been identified, COX-1 and COX-2, which are

encoded by different genes [7]. COX-1 and COX-2 have similar structural and kinetic properties but show distinct cell specific expression and regulation in mammals. COX-1 is expressed constitutively in most cells, whereas the inducible COX-2 form is usually expressed in response to various inflammatory stimuli [8].

COX-1 was initially identified and purified in the 1970s, using classical biochemical techniques, from bovine [9] and sheep [10] vesicular glands and found to be a membrane bound homo-dimer of 70 kDa. The protein contained both the Cyclo-oxygenase and peroxidase activities required to form, respectively, prostaglandin PGG2 and PGH2. Either free or protein-bound heme was required for activity.

Cyclo-oxygenase metabolites are released in high amounts locally at the site of inflammation or systemically after infection. Initially it was believed that this was due to an increase in supply of arachidonic acid. However, in 1990 it was demonstrated that the increase in prostaglandin formation following exposure of isolated cells in culture to inflammatory stimuli was due to an increase in Cyclo-oxygenase enzyme expression [11, Fu, 1990 #5941]. The first step in the formation of prostaglandins is the liberation of arachidonic acid from membrane bound phospholipids. This usually follows the action of phospholipase enzymes, primarily phospholipase A2. Several phospholipase A2 are expressed in a number of different groups, IB, IIA, IIC, IID, IIE, IIF, V, X, XIIA and XIIB. Some PLA2 are present constitutively, and others appear after inflammatory stimuli [12]. We recently reported the expression level of several secreted phospholipases A2 in chickens in case of lung inflammatory disease. Our results showed that in the case of infectious bronchitis, the expression levels of ChPLA2-IB, ChPLA2-IIA, and ChPLA2-X are especially decreased in lungs (the principal target tissue of this infection) by up to 12-, 30- and 1.8-fold, respectively suggesting a down regulation in their expression level. Interestingly, the expression of ChPLA2-V showed a dramatic increase in lungs and spleen. Contrary, no significant differences in the expression level of sPLA2-IIE was observed in uninfected and infected lung and intestine, but its expression was increased significantly in heart, liver and spleen of infected chicken.

The purpose of this paper is to examine the distribution and relative expression of COX-1 and COX-2 in several organs of normal hens and in hens with infection bronchitis.

2. Materials and Method

2.1. Chicken Tissues

Several distinct samples of pancreas, intestine, liver, heart, colon, lung and spleen were collected from both infected and uninfected chicken at the Institute of Tunisian Veterinary Research. The specimens were from 30 to 45 days year-old chickens at the time of diagnosis. The diagnostic was determined according to the pathologic examinations as approved by the Institute of Tunisian Veterinary Research. The histological observations were determined on tissues sections according to the World Health Organization criteria. Several histological normal chicken tissues were collected from animals with no clinical symptoms which were used as controls.

2.2. Histological Analysis

Classical procedure was used for histology. After fixation in Bouin solution, pieces of fixed tissue were embedded into paraffin, cut into 5_m slices and colored with hematoxyline-osine.

2.3. Total RNA Extraction and RT-PCR Amplification of COX-1 and COX-2 mRNA

Total mRNAs were isolated from each chicken tissues sample (100mg) using the single step guanidine isothiocyanate-phenol-chloroform isolation method as described by Chomczynski and Sacchi [3]. Chicken COX cDNA were obtained from total mRNAs by the reverse transcription procedure (Promega). First strand cDNAs were prepared using heat-denaturized (5 min at 70°C) total mRNAs (10 µg) as template, 200 U MMLV reverse transcriptase (Invitrogen), 20 pmol of each deoxynucleoside triphosphate, and 20 pmol of each primer. Reverse transcription was carried out in a total reaction volume of 20 µl for 5 min at room temperature and 60 min at 42°C. The cDNA/RNA heteroduplex was then denaturized at 70°C for 15 min and cooled on ice.

2.4. Quantitative Expression of COX-1 and COX-2 mRNA

Quantitative real time PCR (qPCR) was performed with the Light Cycler 480 system (Roche) using LightCycler FastStart DNA Master SYBR Green I-kit (Roche) according to the manufacturer's instructions. The qPCR conditions were as follows: +95°C for 10min; 45 amplification cycles at +95°C for 10 s, annealing for 10 s (5°C above melting temperature of the primer with lower value), elongation at +72°C for 8s (25 bp/s + 2 s), measurement of fluorescence at +81-90,5°C for 1 s (see Table 1). After amplification, melting curve analysis was performed as follows: +98°C for 10s; annealing temperature for 20s; continuous temperature gradient to +95°C with 5 acquisitions/s. The tissue cDNA samples were diluted 1/ 10 with nuclease free water, and the same cDNA dilution was used for all qPCR reactions. A fresh 10^{-1} to 10^{-4} serial dilution solutions are prepared from each target gene for analysis starting from the 10^{-1} dilution of a purified PCR product.. As housekeeping genes, we measured the mRNA expression levels of chicken β-*actine*. The qPCR primers used are shown in Table 1.

In qPCR, every gene had PCR efficiencies ranging from 1.902 to 1.972, as calculated from the internal standard; thus, no efficiency correlation was performed. The Light Cycler 480 Software with absolute quantification was used to calculate the concentrations and standard deviations of samples was calculated by dividing the concentration of the target gene to the concentration of the housekeeping gene.

The relative expression of the mRNA in the tissue Standard deviations were calculated.

3. Results

3.1. Histology Analysis of Hens Tissues with Inflammatory Disease

To investigate the damages caused by the viral infection on chicken infectious bronchitis, we observed the pulmonary histological changes by microscope. Histological analysis from lung's tissue with infection bronchitis revealed severe lesions consistent with acute pneumopathy, compared to lungs from uninfected animals (Figure 1). There were normal lung tissue structure and clear pulmonary alveoli in the control groups (Figure 1A). Infection with coronavirus in hens caused acute alveolar damage and acute inflammation (Figure 1B). The histopathologic changes of lung included: congestion and edema (pink colored liquid in pulmonary alveoli), the appearance and sequestration of inflammatory cells and the markedly thickened alveolar wall. The lung pathology of hens infected with the pandemic coronavirus have shown primarily diffuse alveolar damage with extensive hyaline membrane formation and intraalveolar edema (Figure 1B). The vessels in alveolar walls show thrombosis, and varying degrees of mixed inflammatory infiltrate (data not shown). Alveolar epithelial cells and pneumocytes show reactive changes, and their nuclei appear enlarged. Intraalveolar hemorrhage and erythrophagocytosis have also been observed.

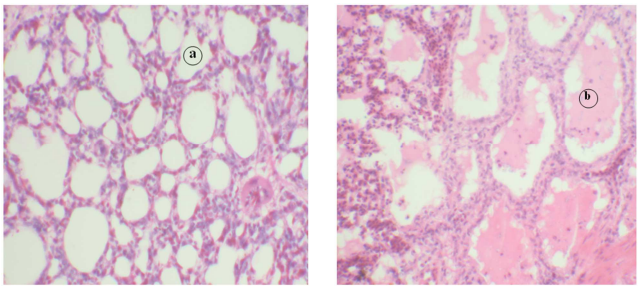

Figure 1. *Histological comparison of the lungs from normal hens controls (A), and from hens with infectious bronchitis (B). a Clear pulmonary alveoli. The histopathologic changes of lung included: diffuse alveolar damage characterized by congestion and intra alveolar edema (pink colored liquid in pulmonary alveoli b), the appearance and sequestration of inflammatory cells and the markedly thickened alveolar wall.*

3.2. Quantification of COX-1 and COX-2 mRNA in Organs from Normal Chickens

Figures 2 and 3 show marked differences between COX-1 and COX-2 expression. The highest relative expression levels of COX-1 was in lungs (6.724) and heart (3.089). Whereas, the lowest relative expression level was observed in the pancreas (1.136) and colon (1.892) and its expression was not detectable in the intestine, liver nor in spleen (figure 2).

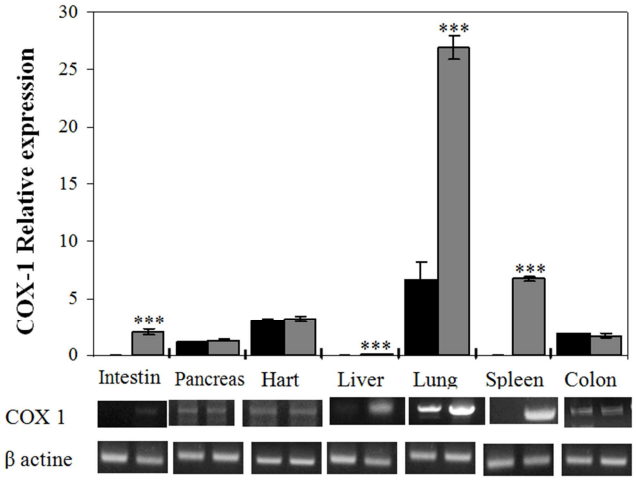

Figure 2. *COX-1 mRNA expression profiles of chicken. Gene-specific primers were used to determine the expression profiles of the avian COX-1. Chicken gene was analyzed twice independently using the LightCycler 480 system. Error bars represent the observed standard deviation. The relative expression values were scaled to a range of 0–30. The values shown were calculated using β-actins as the housekeeping gene. Results are expressed as the mean +/- SEM percentage of the control value (n=3). *p < 0.05, **p < 0.01, ***p < 0.001.*

COX-2 mRNA was quantified with the same way from normal tissue as described for COX-1. Results showed a significant widespread tissue distribution, including lung since its relative expressed level reached 3.544 in small intestine, 2.818 in liver, 1.837 in spleen and 1.17 in heart (Figure 3). Contrary to COX 1, COX-2 was not detectable in pancreas nor in colon of healthy hens (Figure 3).

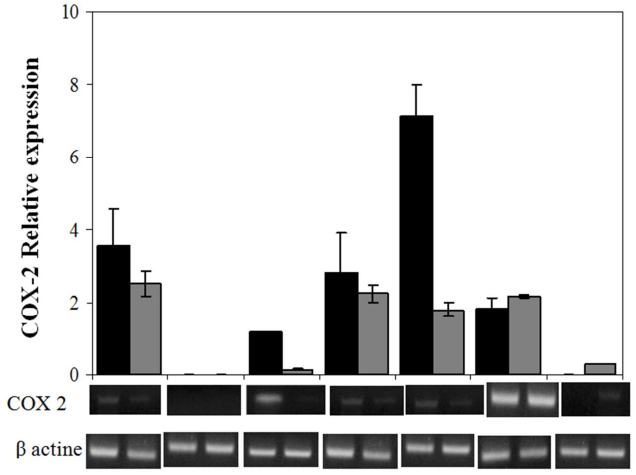

Figure 3. *COX-2 mRNA expression profiles of chicken. Gene-specific primers were used to determine the expression profiles of the avian COX-2. Chicken gene was analyzed twice independently using the LightCycler 480 system. Error bars represent the observed standard deviation. The relative expression values were scaled to a range of 0–10. The values shown were calculated using β-actine as the housekeeping gene. Results are expressed as The mean +/- SEM percentage of the control value (n = 3).*p < 0.05, **p < 0.01, ***p < 0.001.*

3.3. COX-1 Is Upregulated in Avian Lung Inflammation

The variation of the expression level in several tissues associated with the infectious bronchitis disease was

examined to establish possible gene co-regulation (figure 2). Quantitative real-time PCR for COX-1 mRNA expression show significant increase in avian infection bronchitis as compared to normal lungs samples ($P < 0.05$). Interestingly, COX-1 expression level increases, in lungs by up to 4- fold in infected hens. A drastic elevation in the expression level of COX-1 in spleen was also observed compared with normal hens. Meanwhile, a slight increase of COX-1 expression in pancreas by up to 1.16-fold was also observed. Meanwhile, expression of COX-1 gene was detected in the small intestine and liver of healthy subjects but its expression appears high in chickens with infection bronchitis. Whereas, there was no difference in COX-1 expression in inflamed chicken's heart and colon compared to the normal hens.

3.4. COX-2 Is Widely Distributed in the Hen Tissues, But Not Increased in Lung Infection

No significant difference in COX-2 expression was observed in almost all tissues with infection bronchitis compared to normal ones. Thus, if we focus on the mRNA level of COX-2 gene in tissues from normal chickens, we noticed that this enzyme is widely distributed in several tissues including intestine, heart, liver, lung and spleen. In contrast, the expression of this enzyme in the pulmonary infected chicken's tissues was generally very low. The relative expression level was significantly decreased ($p<0.05$) in lungs and heart of infected chickens, by up to 4-fold and 1.4-fold, respectively (figure 3). However, there was no difference in COX-2 expression in the small intestine, pancreas, liver and spleen of normal chicken compared to infected hens. Figure 4 indicates that the ratio of COX-1 from infected tissues to COX-1 control expression was significantly higher in COX-2 samples as compared to normal avian samples ($P < 0.05$).

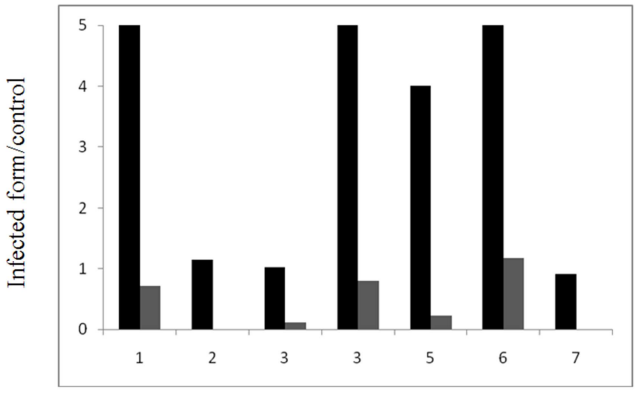

Figure 4. *Ratio of COX-1 (infected hens)/COX-1 (controls) expression in several tissues and COX-2 (infected hens)/COX-2 (controls). Asterisks indicate a significant increase in tissues with inflammation as compared to normal ones ($P < 0.05$). Bars indicate standard error.*

4. Discussion

Avian infectious bronchitis (IB) is one of the most serious diseases of chickens. It is of economic importance in the poultry industry worldwide and is associated with respiratory disease, reduction in weight gain, poor egg production and quality, and decreased feed conversion efficiency.

Its etiologic agent is the avian infectious bronchitis coronavirus (IBV), which is a *Gamma* coronavirus of the *coronavirus* genus and replicates primarily in the upper respiratory tract, kidney, and oviduct of chickens [13-15]. The prevalence and severity of respiratory diseases in commercial chicken flocks has increased recently due to the intensification of the poultry industry. In a previous work, we have analysed variation in the expression pattern of secreted PLA2 genes in the intestine, pancreas, heart, liver, colon, lung and spleen matched from chicken with inflammatory lung disease to establish possible gene co regulation. Our results provided the first comprehensive analysis of the expression pattern of the sPLA2 in normal chicken's organs, as well as in pulmonary infected birds to establish that several sPLA2 may contribute to the progression or modulation of infectious bronchitis in poultry.

Knowing that the first step in the formation of prostaglandins is the liberation of arachidonic acid from membrane bound phospholipid, allows us to examine the distribution and the relative expression of COX-1 and COX-2 in several organs and tissues of normal hens and hens with IB. This can help to gain insight into the origins and causes of avian infection bronchitis first, and to focus on the possible role of COX-1 and COX-2 in avian inflammation second.

Quantitative real-time PCR analysis indicates that COX-1 mRNA is expressed in lungs, heart, pancreas and colon, with relatively different level. Whereas, it was not detected in the small intestine and liver. We noted also a wide distribution of COX-2 in tissues from normal hens, including lung, small intestine, liver, spleen and heart with relative expression level of 7.13, 3.54, 2.81, 1.83 and 1.17, respectively. These results, confirms that Cyclo-oxygenase enzymes with distinct cell-specific expression and regulation are essential for normal physiological processes yet their aberrant expression is a critical factor in a host of pathologies. In mammals, COX-2 is present in various neoplasms, such as colorectal, lung, stomach, pancreas, breast, urinary, bladder and esophageal neoplasms [16]. Whereas, COX-1 is constitutively expressed and related to physiological functions [17].

qPCR gene expression analysis of cyclooxygenases in several tissues from chicken suffering of an infectious bronchitis, indicates that COX-1 mRNA is elevated in hen lungs inflammation compared to normal hen. However, a significant increase was also observed in the small intestine, lungs, pancreas, liver and spleen of infected chicken. These results show for the first time that COX-1 expression is markedly increased in the pulmonary infection. A significant correlation exists between COX-1 expression level and avian infection bronchitis disease. However, no significant correlation was obtained in the case of COX-2. Meanwhile, no difference in COX-2 mRNA expression was observed in liver, small intestine and spleen between normal and infected hen. A significant decrease of its expression level was observed in lungs and heart. Previous work was focused on

determination of tissue prostaglandin concentration, obtained from ovarian tumour [22]. PGE2 and PGE2 metabolites were measured using competitive enzyme immunoassays (EIAs). They showed that COX-1 mRNA expression is significantly higher ($P < 0.05$) in ovarian tumour samples compared to normal ovaries while there is no significant difference in expression of COX-2. Immunohistochemistry studies support this finding and show that COX-1 was expressed only in tumour samples, however COX-2 expression was unchanged [18].

To examine the relative expression of these two COX isoforms, we compared the ratio of COX-1 from infected tissues to COX-1 control in case of inflammation to this ratio of COX-2 (Figure 4). This ratio seems to be higher in COX-1 form compared to COX-2. Although these data are not relevant to the biological activity of either isozyme, they do show that minor differences in COX-2 expression do not alter the main increase of COX-1 expression in infected chicken compared to normal ones (Figure 4).

Furthermore, in our previous work, the expression of chicken sPLA2 has been examined in animals with infectious bronchitis. qPCR experiments revealed that ChPLA2-V is expressed in lungs of infected animals but not in normal ones. One of the most notable functions of ChPLA2-V is its ability to initiate inflammatory response. In fact, following its production by ChPLA2-V, arachidonic acid is metabolised through the cyclooxygenase pathways to yield prostaglandins. As oxygenated metabolites, these eicosanoids promote inflammation through increased vascular permeability and the recruitment of granulocytes such as neutrophils to the site of injury or infection.

Table 1. *primers used for the qPCR analyses (F): forward; (R): reverse.*

Primer name	sequence (5'-3')	PCR product size	Melting temperature	Temperature of fluorescence measurement
COX-1 (f)	TCAGGTGGTTCTGGGACATCA	200	66	87
COX-1 (r)	TGTAGCCGTACTGGGAGTTGAA	200	66	87
COX-2 (f)	CTGCTCCCTCCCATGTCAGA	200	65	83.5
COX-2 (r)	CACGTGAAGAATTCCGGTGTT	200	65	83.5
PLA2 V (f)	AATGCTCTCCTTGCATTGGCCATACTG	200	49	90
PLA2 V (r)	AGCTGGCAGCACTGTCTGTGCATCC	200	49	90
B-Actine (f)	CACAGATCATGTTTGAGACCTT	60	60	85
B-Actine (r)	CACAATACCAGTGGTACG	60	60	85

Abbreviation

cyclo-oxygenase: COX; phospholipase A2: PLA2; prostaglandin: PG; PCR: quantitative qPCR; chicken PLA2: ChPLA2; infectious bronchitis IB.

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