Activation of peroxisome proliferator–activated receptor gamma induces anti-inflammatory properties in the chicken free avian respiratory macrophages

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Abstract

Background: Activation of peroxisome proliferator activated receptor gamma (PPAR γ) in the alveolar macrophages (AM) by selective synthetic PPAR γ ligands, improves the ability of the cells to resolve inflammation. In birds, respiratory macrophages are known as free avian respiratory macrophages (FARM) and show distinct functional differences from AM. The effects of treating FARM with PPAR γ ligands are unclear.

Methods: FARM were harvested by lavage of chicken respiratory tract and their morphology assessed at microscopic level. The effects of PPAR γ agonists on the FARM in vitro viability, phagocytic capacity and proinflammatory cytokine (TNF-α) production were assessed.

Results: FARM had eccentric nucleus and plasma membrane ruffled with filopodial extensions. Ultrastructurally, numerous vesicular bodies presumed to be lysosomes were present. FARM treated with troglitazone, a selective PPAR γ agonist, had similar in vitro viability with untreated FARM. However, treated FARM co-cultured with polystyrene particles, internalized more particles with a mean volume density of 41 % compared to that of untreated FARM of 21 %. Further, treated FARM significantly decreased LPS-induced TNF-α production in a dose dependent manner.

Conclusion: Results from this study show that PPAR γ synthetic ligands enhance phagocytic ability of FARM. Further the ligands attenuate production of proinflammatory cytokines in the FARM, suggesting potential therapeutic application of PPAR γ ligands in the management of respiratory inflammatory disorders in the poultry industry.

Keywords: Avian, Free avian respiratory macrophages, Peroxisome proliferator-activated receptor, Troglitazone
clearance of inflammatory sites in the mammalian lung [11, 12]. Chicken peroxisome proliferator activated receptor gamma (chPPAR γ) is structurally different from the mammalian PPAR γ suggesting different functional roles [13, 14].

Respiratory disease conditions, partly characterized by chronic inflammation of the respiratory epithelia, cause immense economic losses in the poultry industry [15, 16]. Despite the losses, relatively little is known about the avian pulmonary cellular defense mechanisms [17, 18]. In birds, respiratory macrophages are referred to as free avian respiratory macrophages (FARM) [19, 20] and dearth of the cells in the lung air sac system has been purported to foreordain a weak innate immunity thus predisposing birds to respiratory infections [21–23]. However, FARM exhibit a significantly higher phagocytic ability than AM [24] and mobilization of the cells in the avian respiratory system does not occur after intravenous application of lipopolysaccharide, incomplete freunds adjuvant or glucan, compounds known to induce migration of AM from the lung interstitium into the alveolar space [25].

The effects of PPAR γ agonists on FARM are unknown. The aim of this study was, therefore, to determine:

(i) The effect of selective synthetic PPAR γ ligands on the phagocytic capacity of FARM
(ii) The effect of the PPAR γ ligands on proinflammatory cytokine production by assessing TNF-α secretion in lipopolysaccharide activated FARM.

Methods

Pulmonary lavage of the avian respiratory system

All experimental procedures were approved by the Kenyatta University Animal Ethics Committee. FARM were obtained from the respiratory system of mature specimens of domestic fowl as previously described [26]. Briefly, chickens were anesthetized and then euthanized by intravenous injection of an overdose of pentobarbitone sodium (Euthanase®) into the brachial vein. The trachea was then exposed and sterile pre-warmed (40 °C) phosphate buffered saline (PBS) was poured down the respiratory system. Recovered lavage fluid was centrifuged and the pelleted FARM re-suspended in sterile cell-culture medium.

Processing of FARM for transmission electron microscopy (TEM)

Recovered FARM were fixed in 2.5 % phosphate buffered glutaraldehyde solution for 12 h. The cells were then post fixed in 1 % osmium tetroxide in 0.1 M sodium cacodylate buffer followed by dehydration in graded replacement of ethanol (70 %, 80 %, 90 %, and 100 % twice). Gradual replacement of ethanol with propylene oxide was then done before infiltrating and embedding the cells in epoxy resin. Using Reichert® ultra-microtome, semithin and ultrathin sections were obtained from processed blocks. The semithin sections were collected on glass slides and stained with 3 % toluidine blue while the ultrathin sections were picked on copper grids, stained with uranyl acetate and lead citrate, and observed with a Philips 201C TEM under an accelerating voltage of 60 Kv. Micrographs were developed from the processed sections for morphological studies.

In vitro viability of the FARM

FARM were washed three times in PBS and re-suspended at a concentration of 1.5 × 10⁵ cells/ml in sterile eppendorf tubes containing RPMI-1640 cell culture medium and treated with 9 μM of troglitazone (Abcam, Science Park Cambridge, UK) for 1 h. A control pellet of FARM was processed in a similar manner but without troglitazone. The tubes were kept for 4 h in an incubator (40 °C and 5 % CO₂). A viable count of FARM was assessed using trypan blue in hemocytometer.

Phagocytosis assays

Recovered FARM were re-suspended at concentrations of 1.5 × 10⁵ cells/ml in fresh RPMI - 1640 in sterile eppendorf tubes. The FARM were treated with 9 μM of troglitazone in incubator for 1 h. Treated and untreated...
FARM were co-cultured with polystyrene particles (Sigma 3050 Spruce Street, USA) in incubator for 3 h. Shaking of tubes was done regularly to ensure contact of the cells and the particles. Thereafter, FARM were fixed in 2.5 % phosphate buffered glutaraldehyde solution for 12 h and processed for TEM. Semithin and ultrathin sections were processed for estimation of diameter of the FARM and volume density of internalized particles in the cells respectively.

**Estimation of the diameters of the FARM and the volume density of the phagocytized particles**

Diameters of FARM were determined under an ocular graticule with a linear scale at a magnification of × 100. In each field, to avoid bias, only diameters of FARM at the four corners of the fields were measured. The volume density of the phagocytized particles in the FARM was estimated as previously described [27]. Briefly, ultrathin sections were processed and the corresponding micrographs recorded on a 35-mm electron microscope film prior to being projected onto a screen at a final magnification of × 14 000. A quadratic lattice grid was superimposed at a random position onto each projected image. The total number of points falling onto profiles of the phagocytized particles [P (p)] and on entire cell [P (c)] was counted. Volume density of phagocytized particles [V V (p, c)] was then calculated as follows: V V (p,c) = P (p) / P (c)

**Measurement of TNF-α production by the FARM**

The FARM were washed three times in PBS and seeded at a density of 1.5 × 10^5 cells/well in RPMI 1640 with 5 % FCS into 24-well tissue culture. The cells were treated with varying doses (3 μM, 6 μM and 9 μM) of troglitazone for 1 h before addition of 0.1 ng/ml lipopolysaccharide (LPS). After 24 h incubation at 40 °C in 5 % CO₂, the supernatants were harvested for TNF-α measurement using ELISA kit (Bicom Biotech, SA). Briefly, the supernatants were diluted appropriately and incubated with anti-chicken TNF-α antibody coated plate at 40 °C for 1 h. The plate was washed 3 times in phosphate buffered saline-tween (PBS-T) followed by addition of biotin–streptavidin HRP labeled anti-chicken TNF-α. The plate was incubated for 30 min at 40 °C followed by 3 washes in PBS-T before addition of chromogen.

**Data analysis**

For paired experiments, student t–test was used to compare the values on the chicken FARM in the various experiments while analysis of group data for multiple comparisons was performed using ANOVA followed by Duncan’s multiple range test to determine the level of differences. The level of significance was set at $p \leq 0.05$ confidence level. The results were presented in form of tables, graphs and micrographs. Means ± Standard Error

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**Fig. 2** a Photomicrograph (×400) and (b) electron micrograph (×950) of FARM recovered by lavage of the chicken respiratory system. The cells have eccentric nucleus (N) and plasma membrane ruffled with filopodial extensions (arrows). In (b), numerous cytoplasmic vesicular bodies presumed to be lysosomes (red arrows) were identified. The Semithin (1 μm) and the ultrathin (80 nm) sections were cut from processed blocks of epoxy resin embedded cell suspensions.

**Fig. 3** Under similar experimental conditions, there was no significant ($p \geq 0.05$) difference in the in vitro viability of troglitazone treated and untreated FARM. The bars show SEM.
of the Mean (SEM) were used to explain the results in text and tables.

Results
Morphological observations
The lavage fluid recovered from the respiratory system of the chicken contained both ciliated epithelial cells and FARM (Fig. 1). Typically, the FARM had plasma membrane ruffled with filopodial extensions and an eccentrically located nucleus (Fig. 2). Ultra structurally, the FARM had variably electron dense vesicular cytoplasmic organelles presumed to be lysosomes (Fig. 2b).

In vitro viability of the FARM
Troglitazone treatment of chicken FARM at a dosage of 9 μM for 4 h did not compromise FARM viability. Troglitazone treated FARM exhibited equivalent ($p \geq 0.05$) in vitro viability with untreated FARM under similar experimental conditions. The in vitro viability of troglitazone treated FARM and untreated FARM was $82 \pm 1.5\%$ and $83 \pm 2.5\%$ respectively (Fig. 3).

Morphometric observations
The mean diameter of troglitazone treated FARM was not significantly ($P \geq 0.05$) different from that of untreated FARM (Table 1). Quantitative estimation of loading of FARM with polystyrene particles was assessed using micrographs (Figs. 4 and 5). Despite having equivalent diameters and therefore volume, the mean volume density of internalized particles per unit volume of treated FARM was $41 \pm 1.0\%$, a significant ($P \leq 0.05$) value compared to that of the untreated FARM which was $21 \pm 1.1\%$ (Fig. 6).

The effect of troglitazone on TNF-α production by the chicken FARM
To define the functional role of PPAR-γ in the chicken FARM, the effect of troglitazone on cytokine production by the chicken FARM was measured by determining TNF-α concentrations in culture supernatants of lipopolysaccharide-stimulated FARM after treatment with graded (3 μM, 6 μM and 9 μM) doses of troglitazone. Lipopolysaccharide elicited considerable amounts of TNF-α production by FARM at concentration of 0.1 ng/ml. Addition of troglitazone to cultures of LPS-induced chicken FARM, significantly ($p \leq 0.05$) inhibited TNF-α production by the FARM in a dose dependent manner (Fig. 7).

### Table 1 Mean diameter of troglitazone treated and untreated FARM

| Slide Number | Diameter of treated FARM (μm) | Diameter of untreated FARM (μm) |
|--------------|-------------------------------|---------------------------------|
| 1            | 12                            | 14                              |
| 2            | 15                            | 10                              |
| 3            | 12                            | 11                              |
| 4            | 11                            | 12                              |
| 5            | 13                            | 12                              |
| 6            | 10                            | 10                              |
| 7            | 11                            | 13                              |
| 8            | 9                             | 13                              |
| 9            | 14                            | 11                              |
| 10           | 12                            | 12                              |
| 11           | 10                            | 11                              |
| 12           | 12                            | 13                              |
| Mean         | 11.7                          | 11.8                            |
| SEM          | 0.5                           | 0.4                             |

Data represent the mean diameter of troglitazone treated and untreated FARM that were co-cultured with polystyrene particles. The mean diameter of treated FARM was not significantly ($p \geq 0.02$) different from that of untreated FARM.

Fig. 4 Photomicrographs showing internalized polystyrene based particles (arrows) in (a) troglitazone untreated FARM and (b) troglitazone treated FARM (b). The FARM and the particles were co-cultured in RPMI 1640 culture medium for 3 h. The photomicrographs were prepared from semithin (1 μm) sections cut from processed blocks of epoxy resin embedded cell suspension. (x400)
In the present study, we examined the effect of PPARγ ligands on the phagocytic capacity of FARM. This study reports for the first time that the phagocytic capacity of freshly harvested chicken FARM is enhanced by selective synthetic PPARγ ligands. Selective synthetic PPARγ ligands improve the phagocytic ability of AM with subsequent clearance of inflammatory site, an essential process during restoration of alveolar architecture in the mammalian lung [28]. Chronic inflammation, partly characterized by accumulation of FARM with diminished phagocytic ability in the inflammatory site, causes gross respiratory epithelial tissue destruction with subsequent high mortality in the poultry industry [29, 30]. Phagocytosis is the most important defense mechanism in all phyla of the animal kingdom [31] and therefore, up regulation of phagocytic ability of FARM by PPARγ agonists could be critical in clearance of inflammatory stimuli in the avian lung. In this study, PPARγ ligands substantially improved the phagocytic ability FARM. Further, troglitazone treated FARM had irregular vacuoles formed around ingested particles indicating up regulated destruction of internalized particles by the FARM.

A characteristic of non phlogistic phagocytosis is the ability of activated macrophages to clear inflammatory stimuli with diminished production of proinflammatory cytokines [32]. To elucidate the non phlogistic functional role of PPARγ ligands during phagocytosis in the FARM, we treated lipopolysaccharide activated FARM with varying doses of troglitazone. Troglitazone treated FARM inhibited TNF-α production in lipopolysaccharide activated FARM in a dose dependent manner. TNF-α has been reported as the primary regulator of inflammation [33] and activated FARM produce TNF-α in response to respiratory inflictions [34]. However, incessant production of proinflammatory cytokines prolongs inflammation contributing to pathogenesis of respiratory disease conditions such as aspergillosis [35]. An understanding of the
mechanisms that enhance FARM to regulate inflammatory responses may permit development of products for the enhancement productivity in the poultry industry. FARM are the predominant immune cells in the avian lung [36] therefore, synthetic PPAR γ agonists could be used in attenuating proinflammatory cytokine production by the cells as a therapeutic intervention in resolving respiratory inflammatory disease conditions in the poultry industry.

Conclusion

In this study, selective synthetic PPAR γ agonists significantly enhanced the phagocytic index of chicken FARM. Further, the PPAR γ ligands attenuated production of proinflammatory cytokine TNF-α by activated FARM. This study, therefore, concludes that PPAR γ ligands are attractive therapeutic novel drug targets for resolution of avian respiratory inflammatory disease conditions.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

Mutua M. Patrick carried out the experiments and prepared the manuscript as part of his PhD (Immunology) research project. All authors read and approved the final manuscript draft.

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References

1. Geiser M, Baumann M, Cruz-Orive LM, Hof V, Weber U, Gehr P. The effect of particle inhalation on macrophage number and phagocytic activity in the intrapulmonary conducting airways. Am J Respir Cell Mol Biol. 2002;10:594–603.
2. Kumar V, Sharma A. Neutrophils: Cinderella of innate immune system. Int Immunopharmacol. 2010;10:325–34.
3. Yan Y, Gang H, Eran L, Quyue W, Jian K. PPAR Gamma agonists regulate tobacco smoke – induced toll like receptor 4 expression in alveolar macrophages. Respir Res. 2014;15:1–14.
4. Desvergne B, Wahli W. Peroxisome proliferator Activated Receptors: Nuclear control of Metabolism. Endocr Rev. 1999;20:649–88.
5. Klewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, et al. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Press USA. 1994;91:7355–59.
6. Braissant O, Foulle E, Scitto C, Dauca M, Wahli W. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -δ, and γ in the adult rat. Endocrinology. 1996;137:354–66.
7. Chavla A, Schwarz EJ, Dimaculangan DO, Lazar MA. Peroxisome proliferator-activated receptor (PPAR) γ adipose-predominant expression and induction early in adipocyte differentiation. Endocrinology. 1994;135:798–800.
8. Kazuhiro A, Shigekazu S, Takafumi S, Kingo C, Hiroshi N. Antiinflammatory Roles of Peroxisome Proliferator Activated Receptor γ in Human Alveolar Macrophages. Am J Respir Crit Care Med. 2004;169:195–200.
9. Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WD, Willson TM, Kleweer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPAR γ). J Biol Chem. 1995;270:1295–312955.
10. Willson TM, Cobb JE, Cowan DJ, Wiethoe RF, Corrêa ID, Prakash SR, et al. The structure–activity relationship between peroxisome proliferator-activated receptor γ agonism and the antihyperglycemic activity of thiazolidinediones. J Med Chem. 1996;39:685–8.
11. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998;391:82–6.
12. Cather G, Siobhan F, Killeen H, Nicos AP, Nancy H, Hugh RB. Lipoxins Rapidly Stimulate Nonphlogistic Phagocytosis of Apoptotic Neutrophils by Monocyte Derived Macrophages. J Immunol. 2000;164:1663–7.
13. Takada I, Kobayashi M. Structural features and transcriptional activity of PPARs (α, β and γ). PPAR Res. 2013;2012:1–7.
14. Sato K, Fukuoka K, Seki Y, Akiba Y. Expression of the chicken PPAR-gamma gene is influenced by aging, nutrition, and agonist administration. Poult Sci. 2004;83:1342–7.
15. Currie RW. Aspects in poultry: recent investigations. Avian Pathol. 1999;28:313–26.
16. Sultana S, Rashid SMH, Islam MN, Ali MH, Azam MG. Pathological Investigation of Avian Aspergillosis in Commercial Broiler Chicken at Chittagong Agriculture and Veterinary University. Int J Innov Appl Stud. 2015;10:366–76.
17. Golemboski KA, Whelan J, Shaw S, Kinsella JE, Dietert PR. Avian Inflammatory Macrophage Function: Shifts in Achoridonic Acid Metabolism, Respiratory Burst, and Cell Surface Phenotype During Response to Sephadex. J Leukoc. 1999;48:495–501.
18. Brown RE, Brain JD, Wang N. The avian respiratory system: a unique model for studies of respiratory toxicosis and for monitoring air quality. Environ Health Perspect. 1997;105:188–200.
19. Reese SG, Dalaman T, Kapers B. The avian lung – immune system: a review. J Cell Biol. 2006;73:111–24.
20. Fulton RM, Reed WM, Dedicola IB. Light microscopic and ultra-structural characterization of cells recovered by respiratory lavage of 2 and 6 week old chickens. Avian Dis. 1996;48:107–109.
21. Toth TE. Nonspecific cellular defense of the avian respiratory system: a review. Dev Comp Immunol. 2000;24:121–39.
22. Kama SG, Adekunle JS, Maina JN. Comparative in vitro study of interactions between particles and respiratory surface macrophages, erythrocytes, and epithelial cells of the chicken and the rat. J Anat. 2008;213:452–63.
23. Maina JN, Cowley HM. Ultra-structural characterization of the pulmonary cellular defenses in the lung of a bird, the rock dove, Columba livia. Royal Society London. 1998;265:1567–72.
24. Mutua PM, Gicheru MW, Makanya AN, Kama SG. Comparative quantitative and qualitative attributes of free surface respiratory macrophages in the duck and rabbit. Int J Morphol. 2011;2:353–62.
25. Nganpiep L, Maina JN. Composite cellular defense stratagem in the avian respiratory system: functional morphology of the free surface macrophages and specialized pulmonary epithelia. J Anat. 2002;200:499–516.
26. Toth TE, Siegel PB. Cellular defense for the avian respiratory tract: paucity of free residing macrophages in the normal chicken. Avian Dis. 1986;30:67–75.
27. Gundersen HJG. Notes on the estimation of the numerical density of arbitrary profiles: the edge effect. J Microsc. 1977;111:219–23.
28. Krey G, Braissant O, L’Horest H, Kahlhoeven E, Perroud M, Parker MG, et al. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol Endocrinol. 1997;11:779–91.
29. Nagy L, Tontonoz P, Alvarez JCA, Chen H, Evans RM. Oxidized LDL regulates monocyte inflammatory cytokines. Nature. 1998;391:82–6.
30. Currie RW. Aspects in poultry: recent investigations. Avian Pathol. 1999;28:313–26.
31. Peter KKW, Ian KC, Paul JE, Mathias E, Ian PW. The Role of the Interleukin-6 Family of Cytokines in Inflammatory Arthritis and Bone Turnover. Arthritis Rheum. 2003;48:1177–89.
33. Zhang SP, Lillehoj HS, Ruff MD. In vivo role of tumor necrosis like factors in Eimeria tenella infection. Avian Dis. 1995;39:859–66.
34. Klasing K. Avian inflammatory response mediation by macrophages. Poult Sci. 1991;70:1176–86.
35. Pascal A, Simon T, Dongying W, Manjula D, Guillaume L. Aspergillus fumigates in poultry. Int J Microbiol. 2011;2011(10):1–14.
36. Maina JN. Some recent advances on the study and understanding of the functional design of the avian lung: morphological perspectives. Biol Rev Camb Philos Soc. 2002;77:97–152.

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