Brain IL-6- and PG-dependent actions of IL-1β and lipopolysaccharide in avian fever

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BIRDS, LIKE MAMMALS, DEVELOP FEVER when exposed to viruses and bacteria (25). The physiological mechanism responsible for febrile mediation in birds has not been elucidated. In homeothermic animals, thermoregulation is controlled by the central nervous system; therefore, fever, a nonthermal modifier of thermoregulation, requires adjustment of thermoregulatory controllers in the brain (35, 46). The hypothalamus, with its temperature-sensitive neurons, serves as the dominant controller of temperature regulation in mammals, whereas the rostral brain stem is thought to house neurons that govern body temperature in birds (46). Despite these phylogenetic differences, it is clear that, in mammals and in birds, peripheral activation of the innate immune system results in physiological modifications at the site of thermoregulatory control.

In mammals, proinflammatory cytokines, specifically IL-1β and IL-6, are known to act as mediators between the detection of an infectious stimulus by immune cells in the periphery and the stimulation of fever (5, 35–37). IL-1β is thought to activate IL-6, and circulating IL-6 concentrations are correlated with the upregulated expression of PG-synthesizing enzymes in the periphery and in brain vasculature (5, 34, 49). Humoral PGE2, produced by macrophages of the lungs and liver, was shown to initiate the first febrile phase in mammals (49), while subsequent febrile phases are thought to be mediated by an upregulated expression of PGE2 in the brain itself as a result of the initiation of cyclooxygenase (COX)-2 and microsomal PGE synthase-1 in endothelial cells (34) and the cerebral microvasculature (39). IL-6 likely initiates the synthesis of PGs in the mammalian brain by 1) acting on cells comprising the organum vasculosum laminae terminalis and the subfornical organ (17, 37, 2) crossing the blood-brain barrier (BBB) (3, 4), or 3) instigating the de novo synthesis of proinflammatory cytokines in the brain by binding to endothelial cells of the BBB (20), instigating PG synthesis. PGs, in turn, inhibit warm-sensitive neurons, and, in this way, heat production/retention ensues (5, 26, 33).

Studies investigating the role of these biomolecules in the febrile mechanism of birds have rendered contradictory results, and neither the involvement of PGs nor the function of proinflammatory cytokines has been confirmed. With regard to PGs, intracerebroventricular injection of PGE1 and PGE2 induced fever in fowls and broilers (23, 30, 31) and young chicks (1, 2) at thermoneutrality, but intracerebroventricular injection of PGF2α and PGE2 decreased the body temperature (Tb) of fowls and pigeons, respectively (31, 32). The PG synthesis inhibitor indomethacin abolished LPS-induced fever in broilers (10 mg/kg iv) (23) but only reduced the duration of fever in young chicks (50 mg/kg iv) (2). Furthermore, indomethacin had no effect on the rising phase of fever in pigeons (10 mg/kg iv or 100 μg/kg iv) (32), whereas another PG synthesis inhibitor, diclofenac (5 and 15 mg/kg ip), reduced LPS-induced fevers in Pekin ducks (14). Fraifeld et al. (13) did not detect an increase in PGE2 in the brain tissue of febrile chickens, and Nomoto (32) reported that, in pigeons, LPS seems to stimulate PG synthesis at peripheral sites only. It is clear that a contribution of PG to avian fever requires clarification, because PGs are known to regulate many physiological functions in birds, including circulation and respiration, which are closely related to thermoregulation (21, 48).

Studies investigating a role for IL-6 in the avian febrile response have also rendered inconsistent results. Xie et al. (54) found, in chickens, that LPS (5 mg/kg) caused fever that peaked 3 h postinjection, while plasma IL-6 concentrations were elevated 12 and 24 h after LPS treatment. In contrast, DeBoever et al. (9) found, in chickens, that plasma IL-6 concentrations peaked after 3 h and returned to baseline 9 h after LPS treatment (1 mg/kg), while Tb reached a peak after ~8 h and resolved 24 h after LPS treatment. Although these data suggest that the time course of plasma IL-6 is not correlated with the time course of fever in chickens, it is known that birds possess well-developed cytokine systems (10, 19, 47, 53) and that avian heterophils synthesize (40, 42, 43) and secrete...
(27, 40) proinflammatory cytokines into the circulation after an immune challenge.

So, despite the ambiguous results regarding the involvement of IL-6 and PGs in avian fever, it is reasonable to hypothesize that birds, like mammals, employ proinflammatory cytokines and PGs to mediate fever (14, 18). As thermoregulatory control in birds is positioned within the brain, an investigation of the central effects of proinflammatory cytokines on avian thermoregulation is appropriate. We opted to examine the effect of proinflammatory cytokines on thermoregulation in birds by injecting avian IL-1β and avian IL-6 into the third cerebral ventricle of conscious Pekin ducks (Anas platyrhynchos). In addition, we investigated the role of endogenous IL-6 and PGE$_2$ in the fever response to the bacterial endotoxin LPS by measuring their change following LPS.

We chose Pekin ducks as experimental animals, because the thermoregulatory system of ducks has been characterized and a large amount of information on the central control of Tb is available (46). Pekin ducks are also an appropriate representative of the avian class, because they are gallinaceous, a group thought to be the basal lineage from which all modern-day birds evolved (51).

METHODS

Ethical Approval

The procedures of this study were approved by the Animal Ethics Committee of the University of the Witwatersrand (approval no. 2010/08/04).

Animals

Twenty Pekin ducks (8 male and 12 female), from different flocks, were obtained from a commercial poultry farm when they were 6 mo old, and experimentation commenced after a period of acclimatization, when the birds were ~7 mo old. The ducks had a mean body mass of 2.4 ± 0.4 kg. They were housed together in an indoor room with ad libitum access to water and dry chicken food enriched with minerals and vitamins and were exposed to a 12:12-h light-dark cycle with lights on at 6 AM and an ambient temperature of 22 ± 1°C, which falls within the thermoneutral zone of Pekin ducks (28, 45).

The ducks were randomly assigned to two groups that were used for two series of experiments. In series 1, eight ducks (3 male and 5 female) were used in experiments to clarify the role of IL-1β and IL-6 in avian fever. In series 2, 12 ducks (5 male and 7 female) were used in experiments to clarify the role of PGE$_2$ in LPS-induced fevers in birds. Six ducks (3 male and 3 female) were randomly chosen for the experimental group, and the remaining six ducks (2 male and 4 female) were assigned to the control group.

T$_b$ Measurement

T$_b$ of the ducks was recorded every 10 min with abdominally implanted miniature temperature data loggers (Tidbit, Onset Computer, Bourne, MA) that were calibrated against a high-accuracy thermometer (Quat 100, Heraeus, Hanau, Germany) to an accuracy of 0.1°C and coated with wax (Sasol Wax, Sasolburg, South Africa) before implantation.

Intracranial Cannula

In series 1, implantation of an intracranial cannula into the third ventricular space of the ducks was followed by abdominal implantation of temperature data loggers in one surgical procedure.

Surgical Procedures

For surgical implantation procedures, the ducks were anesthetized with propofol (5 mg/kg iv; Ascent Scientific, Bristol, UK). An endotracheal tube (33 mm ID) was positioned in the trachea to secure the duck’s airway, and anesthesia was maintained throughout the procedure with 2% isoflurane in inspired oxygen (Safe Line Pharmaceuticals, Wadeville, Johannesburg, South Africa). Implantation of the intracranial cannula. A 20-gauge stainless steel guide cannula (Plastics One) was implanted into the third cerebral ventricle of the ducks. The skin over the skull was cut sagittally, and the skull was exposed. A diamond-head dental burr (16-gauge diameter; SS White Burs) was used to drill a hole through the skull immediately above the third cerebral ventricle. The position of the third ventricle in Pekin ducks was mapped in a pilot study and was found to be 18 mm inferior to the base of the skull, medially in line with the sagittal suture and transversely in line with the outer edge of the orbital ridge. The burr was removed before it pierced the dura. We used the tip of a sterile 23-gauge needle as a stylet to pierce the dura and guide the cannula through the dura. The cannula was pushed inferiorly to reach the ventricle. Once we saw clear cerebrospinal fluid (CSF) emerging from the cannula, we closed the opening of the guide cannula with a dummy cap (Plastics One) and secured the cannula in position with three titanium orthopedic screws (Selective Surgical, Johannesburg, South Africa) and cranioplastic cement (Paladur, Heraeus). After the cement had dried, the surgical cut was sutured in such a way that it fit closely around the base of the guide cannula.

Implantation of temperature data loggers. The abdominal feathers were plucked, and a 5-cm longitudinal incision, extending from the apex of the duck’s keel, inferiorly, was made in the belly. A wax-coated temperature data logger (Tidbit) was pushed through the opening created by the incision into the abdominal cavity. Thereafter, the surgical wound was closed with absorbable sutures.

At the end of the surgical procedure, the endotracheal tube was removed, and the ducks were allowed to regain full consciousness before they were released in their housing pen.

Validation of Correct Placement of the Intracranial Cannula

Although we observed CSF emerging from the intracranial cannula during every surgical implantation, we verified the cannula’s position within the third cerebral ventricle of conscious Pekin ducks 3 wk postsurgery with an osmoregulatory study. Artificial CSF (aCSF; 0.8 g NaCl, 22.0 mg KCl, 25.0 mg MgCl$_2$·6 H$_2$O, 29.0 mg CaCl$_2$·6 H$_2$O, 0.1 mL of 0.05 M phosphate buffer, pH 7.37–7.39, in 100.0 ml of distilled water), made hypertonic (400.0 mosmol/kgH$_2$O) by addition of NaCl, is known to activate the release of antidiuretic hormone (arginine vasotocin in birds) and cause antidiuresis when injected intracerebroventriculatly (44). We thus tested the cannula placement by injecting hypertonic aCSF into the cannula and measuring urine output. The ducks were placed into a custom-made cotton sling, which prevented the body from rotating but allowed free movement of the neck, legs, and feet. The legs were loosely tied to the frame of the sling to keep them still, and one of the leg veins was cannulated. Hypotonic saline (200.0 mosmol/l) was infused intravenously into the leg vein at a rate of 1 ml/min with an infusion pump (Perfusor, B. Braun, Melsungen, Germany). Urine collection was started after 3 h of continuous hypotonic saline infusion to prevent fecal contamination. Urine was collected through a perforated Perspex bulb inserted into the cloaca. The bulb was connected via a tube to a compressor, which induced a mild suction. Once collection started, the urine was continuously aspirated into a preweighed glass beaker. When each duck had attained a steady urine output, we recorded the volume of urine excreted at 5-min intervals for 20 min. We then inserted an injection stylet into the intracerebroventricular guide cannula, so that its tip extended 0.1 mm beyond the tip of the guide cannula. We connected the injection syringe to a gas-tight syringe (Hamilton) and injected hypertonic or isotonic aCSF at a rate of 20.0...
μl/min for 5 min. After the injection, the stylet was removed and replaced with the dummy cap. Renal output was recorded at 5-min intervals, starting from the time when the injection stylet was removed, for 20 min posttreatment. The study was randomized, and each duck was given an injection of hypertonic, as well as isotonic, aCSF ≥7 days apart. All eight ducks responded with antidiuresis after hypertonic aCSF administration and were used in subsequent experiments.

Treatments and Treatment Protocols

Series 1: clarifying the role of IL-1β and IL-6 in fever. Ducks were given the experimental and control treatments described in Table 1. Recombinant biologically active chicken IL-1β in supernatant was kindly donated by Prof. Peter Stäheli (Department of Virology, Institute for Medical Microbiology and Hygiene, University Medical Clinic, Freiburg, Germany). For the recombinant chicken IL-1β, human 293T cells were transfected with the genetic expression construct of chicken IL-1β. These cells then produce chicken IL-1β, which can be harvested from the cell culture supernatant. It was previously shown that these transfected cells produce biologically active chicken IL-1β (52). Because the IL-1β was in the supernatant of a cell culture of human 293T cells, we used the supernatant of regular human 293T cell culture maintained in DMEM (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum as our control for all IL-1β/ES9252 regular human 293T cell culture maintained in DMEM (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum as our control for all IL-1β functions.

Table 1. Experimental and control treatments and treatment combinations

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experimental</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.0 ng</td>
<td>icv</td>
</tr>
<tr>
<td></td>
<td>10.0 ng</td>
<td>icv</td>
</tr>
<tr>
<td></td>
<td>100.0 ng</td>
<td>icv</td>
</tr>
<tr>
<td>IL-6</td>
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<td>icv</td>
</tr>
<tr>
<td></td>
<td>1.2 μg/kg</td>
<td>icv</td>
</tr>
<tr>
<td></td>
<td>2.5 μg/kg</td>
<td>icv</td>
</tr>
<tr>
<td>IL-1β</td>
<td>100.0 ng</td>
<td>icv</td>
</tr>
<tr>
<td>+ Diclofenac</td>
<td>15.0 mg/kg</td>
<td>po</td>
</tr>
<tr>
<td>+ Anti-IL-6</td>
<td>3.0 μg/kg</td>
<td>icv</td>
</tr>
<tr>
<td>LPS</td>
<td>100.0 μg/kg</td>
<td>im</td>
</tr>
<tr>
<td>+ Anti-IL-6</td>
<td>3.0 μg/kg</td>
<td>icv</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant + aCSF</td>
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<td>icv</td>
</tr>
<tr>
<td>aCSF</td>
<td></td>
<td>icv</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td>icv</td>
</tr>
<tr>
<td>+ Saline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>15.0 mg/kg</td>
<td>po</td>
</tr>
<tr>
<td>+ Diclofenac</td>
<td>100.0 ng</td>
<td>icv</td>
</tr>
<tr>
<td>+ aCSF</td>
<td>3.0 μg/kg</td>
<td>icv</td>
</tr>
<tr>
<td>Anti IL-6</td>
<td></td>
<td>icv</td>
</tr>
<tr>
<td>+ Supernatant</td>
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<td>im</td>
</tr>
<tr>
<td>LPS + aCSF</td>
<td></td>
<td>icv</td>
</tr>
<tr>
<td>Saline</td>
<td></td>
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</tr>
<tr>
<td>+ Anti-IL-6</td>
<td>3.0 μg/kg</td>
<td>icv</td>
</tr>
</tbody>
</table>

All doses were made up to 1 ml. For IL-1β, doses are ng per bird. Supernatant was supernatant of regular 293T cell culture maintained in DMEM and 10% fetal bovine serum. aCSF, artificial cerebrospinal fluid; anti-IL-6, antibody to IL-6.

PGE2 Analyses

The PGE2 levels of CSF samples were determined by a commercially available enzyme immunoassay (DetectX, Arbor Assays, Ann Arbor, MI). The instructions of the manufacturers were followed for analysis. All samples were run in triplicate.

Data Analysis

All statistical analyses were performed using the statistical software program GraphPad Prism 5. All data are reported as means ± SD. P < 0.05 was considered statistically significant.

Validation of correct placement of the intracranial cannula. Repeated-measures two-way ANOVA followed by Bonferroni’s post hoc tests was used to compare the average urine flow rate (ml/min) for the ducks immediately before and for 20 min after intracerebroventricular injection of hypertonic or isotonic aCSF.

Tb. We compared the mean ± SD of normal Tb of male and female ducks with a two-way ANOVA. In addition, using a two-way ANOVA, we compared the mean ± SD of Tb responses recorded in male and female ducks, following injection of pyrogens, to ascertain whether there were sex differences in Tb responses. (The Tb responses following the different doses of each pyrogen were analyzed individually.)
For each duck, the Tb responses evoked by the different treatments or treatment combinations given in Table 1 were compared using a two-way ANOVA followed by Bonferroni’s post hoc tests. The results of the two-way ANOVA were used to identify/calculate 1) the latency to fever, 2) the duration of fever, and 3) the peak of fever.

The latency to fever was considered the time period from injection until the first recording of Tb evoked by a treatment that was significantly higher than the Tb evoked by the corresponding control treatment.

The duration of fever was considered the time period from the first to the last recording of Tb evoked by a treatment that was significantly higher than the Tb evoked by the corresponding control treatment.

The peak of fever was the highest Tb evoked by a treatment that was significantly higher than the Tb evoked by the corresponding control treatment.

Since each bird received each treatment, the mean ± SD for the latency, duration, and peak of fever was calculated for the group. The characteristics of fever responses evoked by the different doses of the different treatments were then compared using repeated-measures one-way ANOVA and Bonferroni’s post hoc tests when the ANOVA indicated significant differences between means.

We also calculated the mean ± SD of the Tb recorded in our group of ducks for every 10-min time interval following every treatment. The mean ± SD of the Tb values was then analyzed for an appropriate period of time postinjection and compared between treatments and with the Tb for the corresponding time on a day when the ducks were not handled or subjected to experimentation (“normal” Tb) with a two-way ANOVA followed by Bonferroni’s post hoc tests when the ANOVA indicated significant differences between means. In addition, we calculated differential temperature response indexes (dTRIs) (22) for 32 h after injection of IL-1β and IL-6 and for 16 h after injections containing LPS. For dTRIs following IL-1β and IL-6, we subtracted, for each duck, the Tb recorded at every 10-min interval following injection of the control aCSF from the Tb recorded for the corresponding 10-min interval following injection of IL-1β or IL-6. For dTRIs following treatments containing LPS, we subtracted, for each duck, the Tb recorded for a corresponding time on a day when ducks were not handled or treated (normal Tb) from the Tb following LPS. The differences were summed and integrated to obtain a dTRI. Since each bird received each treatment, the mean ± SD was calculated for the group. For differences in responses to different treatments, the dTRIs were analyzed within and between groups using repeated-measures ANOVA and Bonferroni’s post hoc tests when the ANOVA indicated significant differences between means.

For each sample, we used 150 min after LPS or saline were administered from the time of treatment until euthanasia and compared between treatment groups or treatment combinations given in Table 1 were compared using a two-way ANOVA followed by Bonferroni’s post hoc tests when the ANOVA indicated significant differences between means.

The antidiuresis was initiated within 5 min of hypertonic aCSF injection, and the data were pooled for further analysis.

The peak of fever was considered the time period from injection until the first recording of Tb evoked by a treatment that was significantly higher than the Tb evoked by the corresponding control treatment.

The peak of fever was the highest Tb evoked by a treatment that was significantly higher than the Tb evoked by the corresponding control treatment.

We also calculated the mean ± SD of Tb recorded in the group of ducks for every 10 min following every treatment. The mean ± SD of Tb values was then analyzed for an appropriate period of time postinjection and compared between treatments and with the Tb for the corresponding time on a day when the ducks were not handled or subjected to experimentation (“normal” Tb) with a two-way ANOVA followed by Bonferroni’s post hoc tests when the ANOVA indicated significant differences between means.

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PGE2 analysis. The mean value for each sample was calculated and used for further analysis. We compared the mean ± SD of PGE2 levels in the CSF of male (n = 3) and female (n = 3) ducks treated with LPS, as well as the PGE2 levels in the CSF of male (n = 2) and female (n = 4) ducks treated with saline, using a Student’s t-test for each comparison. We also pooled the data for the sexes and compared the mean ± SD of PGE2 levels in the CSF of ducks treated with LPS and saline using a Student’s t-test.

RESULTS

Validation of Correct Placement of the Intracranial Cannula

Before the intracerebroventricular injection of hypertonic or isotonic aCSF, the ducks attained a steady urine output of 0.91 ± 0.08 ml/min. The injection of isotonic aCSF did not affect urine output (P > 0.05), while injection of hypertonic aCSF caused a significant reduction in urine output in every duck. The antidiuresis was initiated within 5 min of hypertonic aCSF injection and lasted until 15–20 min posttreatment, when urine output started to increase [main effect of treatment: F2,84 = 64.03, P < 0.001; main effect of time: F3,84 = 8.31, P < 0.001; interaction: F6,84 = 3.36, P = 0.005 (by repeated-measures 2-way ANOVA and Bonferroni’s post hoc tests); n = 8].

Tb

Male and female ducks had similar normal Tb and similar Tb responses to pyrogens [main effect of sex: F1,432 = 0.60, P = 0.44 for normal Tb; F1,1,614 = 2.82, P = 0.09 for 2.5 μg/kg IL-6; F1,1,614 = 2.89, P = 0.08 for 1.2 μg/kg IL-6; F1,1,614 = 1.37, P = 0.24 for 0.6 μg/kg IL-6; F1,1,614 = 0.48, P = 0.47 for 100 ng/kg IL-1β; F1,1,614 = 1.25, P = 0.26 for 10 ng/kg IL-1β; F1,1,614 = 2.39, P = 1.22 for 1 ng/kg IL-1β (by 2-way ANOVA)], and the data were pooled for further analysis.

Normal Tb. On days when the ducks were not subjected to experimental procedures, their average Tb was 41.1 ± 0.2°C and 40.8 ± 0.3°C during light and dark hours, respectively. When they were handled to receive treatments, the ducks developed a stress hyperthermic response that did not differ in magnitude between treatments [P = 0.31 (by repeated-measures 2-way ANOVA); n = 8].

Tb following control treatments. Intracerebroventricular injection of aCSF, intracerebroventricular anti-IL-6 + intracerebroventricular aCSF, intramuscular saline, or oral diclofenac + intracerebroventricular aCSF had no effect on normal Tb [main effect of treatment: F2,410 = 1.81, P = 0.14; main effect of time: F204,4100 = 1.24, P = 0.01; interaction: F612,4100 = 0.51, P = 0.98 (by repeated-measures 2-way ANOVA); n = 8].

Tb following intracerebroventricular injections of IL-1β. All three doses of IL-1β (100, 10.0, and 1.0 ng per bird) evoked Tb values that were significantly higher than those following control aCSF. Tb started to rise ~1 h after intracerebroventricular injection, after the stress hyperthermia had resolved [main effect of treatment: F2,5380 = 1070, P < 0.001; main effect of time: F250,5380 = 32.44, P < 0.001; interaction: F804,5380 = 4.32, P < 0.001 (by repeated-measures 2-way ANOVA); n = 8]. The latency, from injection to start of fever, did not differ significantly between doses [P = 0.18 (by repeated-measures 1-way ANOVA); n = 8], the duration of fever was significantly longer following an injection of 100.0 ng of IL-1β than the two smaller doses [t4 = 8.6, P < 0.001 for 100.0 vs. 10.0 ng; t4 = 17.1, P < 0.001 for 100.0 vs. 1.0 ng (by repeated-measures 1-way ANOVA and Bonferroni’s post hoc tests); n = 8], and the peak of fever was significantly higher at 100.0 than 1.0 ng of IL-1β (t5 = 4.4, P = 0.01) and at 10.0 than 1.0 ng of IL-1β [t5 = 3.6, P = 0.02 (by repeated-measures 1-way ANOVA and Bonferroni’s post hoc tests); n = 8].

In ducks, dTRIs evoked by 100.0 and 10.0 ng of IL-1β were significantly higher than that evoked by 1.0 ng of IL-1β [t5 = 5.7, P = 0.002 for 100.0 vs. 1.0 ng; t5 = 3.4, P = 0.02 for 10.0 vs. 1.0 ng (by repeated-measures 1-way ANOVA and Bonferroni’s post hoc tests); n = 8]. Figure 1A shows the profile, peak temperature, fever duration, and dTRI following each dose of IL-1β.

Tb following intracerebroventricular injections of IL-6. Tb values evoked by 2.5 and 1.2 μg/kg of IL-6 were significantly higher than Tb evoked by control injection of aCSF for ~32 h.
Fig. 1. Profiles of core body temperatures (Tb), peak Tb, duration of fever, and differential temperature response index (dTRI) of ducks injected intracerebroventricularly with IL-1β (A) and IL-6 (B). In the Tb profile, arrow indicates time of injection, bars on x-axis show light and dark hours, and solid line shows mean “normal” Tb of ducks. aCSF, artificial cerebrospinal fluid. Values are means ± SD (n = 8). *Significantly different from the other dose(s) (to which it is linked with the bracket) within the group [P < 0.05 (by repeated-measures 1-way ANOVA and Bonferroni’s post hoc tests)]. #P < 0.05.

[main effect of treatment: \(F_{4,5380} = 1,433, P < 0.001\); main effect of time: \(F_{268.5380} = 11.26, P < 0.001\); interaction: \(F_{804.5380} = 2.12, P < 0.001\) (by repeated-measures 2-way ANOVA and Bonferroni’s post hoc tests); n = 8]. Tb appeared to increase after treatment with 0.6 μg/kg of IL-6, but Bonferroni’s post hoc tests, following repeated-measures two-way ANOVA, identified no significant differences in Tb between this treatment and the control injection at any time point. Since 0.6 μg/kg of IL-6 clearly did not evoke fever in our birds, we calculated neither the latency nor the duration of the fever response for this treatment. IL-6 at 1.2 and 2.5 μg/kg evoked an almost immediate increase in Tb, as the first Tb recorded posttreatment was significantly higher than the Tb recorded at treatment and the control injection at any time point. Since 0.6 μg/kg of IL-6 clearly did not evoke fever in our birds, we calculated neither the latency nor the duration of the fever.

The duration of the fever evoked by 2.5 μg/kg of IL-6 was significantly longer than that evoked by 1.2 μg/kg of IL-6 [\(t_8 = 2.3\) and \(t_8 = 0.53, P = 0.07\) and \(P = 0.62\) for dTRI and peak Tb, respectively (by repeated-measures 1-way ANOVA); n = 8]. Tb following intracerebroventricular IL-1β and intracerebroventricular antibodies to IL-6 or oral diclofenac. To ascertain whether IL-1β is pyrogenic because it initiates the synthesis and release of IL-6/PG in the brain of ducks, we gave ducks an intracerebroventricular injection of anti-IL-6 or an oral dose of 15.0 mg/kg of diclofenac and then an intracerebroventricular injection of 100.0 ng of IL-1β. Both treatment combinations, IL-1β + anti-IL-6 and IL-1β + diclofenac, abolished the fever that was evoked by intracerebroventricular IL-1β + oral saline or intracerebroventricular IL-1β + aCSF. At no time posttreatment did these treatments evoke Tb values that differed significantly from the control treatments or normal Tb [main effect of treatment: \(F_{5,5380} = 2.340, P < 0.001\); main effect of time: \(F_{249.5380} = 17.35, P < 0.001\); interaction: \(F_{747.5380} = 6.65, P < 0.001\) (by repeated-measures 2-way ANOVA and Bonferroni’s post hoc tests); n = 8]. Figure 1B shows the profile, peak temperature, fever duration, and dTRI following each intracerebroventricular dose of IL-6.

The key findings of our statistical comparisons between treatments were as follows: 1) the latency to start of fever was significantly shorter for IL-6 than IL-1β [\(t_8 = 2.12, P < 0.001\) (by repeated-measures 1-way ANOVA and Bonferroni’s post hoc tests); n = 8], and 2) the dTRI and the peak Tb evoked by the higher doses of IL-1β and IL-6 were similar [\(t_8 = 2.3\) and \(t_8 = 0.53, P = 0.07\) and \(P = 0.62\) for dTRI and peak Tb, respectively (by repeated-measures 1-way ANOVA); n = 8].
PROINFLAMMATORY CYTOKINES MEDIATE AVIAN FEVER

Changes in Central PGE$_2$ During Fever

As in all the experiments reported here, the ducks developed a stress hyperthermic response when caught for treatment. At ~70 min posttreatment, the $T_b$ values measured in ducks given LPS were not significantly different from those in ducks given a saline injection or from the normal $T_b$ at that time of day ($P = 0.13$ (by 2-way ANOVA); $n = 12$). At 120 min posttreatment, the $T_b$ values in the ducks given LPS became significantly higher than those in the ducks given saline and remained higher until the ducks were killed at 150 min postinjection ($P > 0.05$ (by 2-way ANOVA and Bonferroni’s post hoc tests); $n = 12$; Fig. 4A).

**PGE$_2$ Analysis**

There were no sex differences with regard to the level of PGE$_2$ in the CSF of male and female ducks ($t_4 = 0.61$, $P = 0.55$ for ducks given LPS; $t_4 = 0.65$, $P = 0.55$ for ducks given saline), and the data were pooled for further analysis. Among the ducks killed at 150 min, there was no difference in the level of PGE$_2$ in the CSF between those given LPS and those given saline ($t_{14} = 0.44$, $P = 0.67$ (by Student’s $t$-test); Fig. 4B).

**DISCUSSION**

Fever requires adjustment of the thermoregulatory balance to favor a higher-than-normal $T_b$ (35, 46). Proinflammatory cytokines, specifically IL-1$\beta$ and IL-6, as well as PGs, are thought to be responsible for adjustments in thermoregulatory control in mammals (5, 6, 36–38). One previous study, completed before the identification of chicken IL-1$\beta$, showed that intracerebroventricular administration of recombinant human IL-1$\beta$ caused fever in broiler chickens (23). Our study is the first to investigate a role for proinflammatory cytokines in the

Fig. 3. Left: profile of $T_b$ of ducks injected with LPS (100.0 $\mu$g/kg im) + intracerebroventricular aCSF, intramuscular LPS + antibodies to IL-6 (3.0 $\mu$g/kg icv), and antibodies to IL-6 (3.0 $\mu$g/kg icv) + intramuscular saline. Solid line shows mean “normal” $T_b$. Arrow indicates time of injection, vertical gray bar shows stress hyperthermic response of ducks when they are caught for treatment, and bars on x-axis show light and dark hours. Right: dTRIs evoked by different treatments. Values are means ± SD ($n = 8$). *Significant difference between treatments ($P < 0.05$ (by repeated-measures 1-way ANOVA)).
thermoregulatory control of the avian febrile response using avian cytokines.

Effect of Central IL-1β and IL-6 on Tb

From our results, it is clear that exogenous avian IL-1β and avian IL-6 are pyrogenic within the brain of Pekin ducks. Although both of these cytokines initiated long-lasting fevers with high febrile peaks, IL-6 triggered an almost immediate rise in Tb, whereas IL-1β exerted its pyrogenic action significantly later (~80 min posttreatment). IL-1β appears to be an intermediary in the avian febrile mechanism, positioned prior to the rise of IL-6, and it is plausible that IL-1β initiates the synthesis and release of, or activates, IL-6 within the avian brain. This premise is supported by our results showing that IL-1β-induced fever could be abolished by intracerebroventricular injection of anti-IL-6. Moreover, IL-1β was not pyrogenic in the absence of IL-6 signaling. In mammals, IL-1β and IL-6 are thought to act synergistically in the brain to induce fever (6, 16). Chai et al. (7) showed that, in mice, central IL-6 is an essential febrile mediator, positioned downstream from peripheral and central IL-1β. Febrile mediation in birds seems to be similarly organized; our results clearly show that IL-6 is very pyrogenic when injected into the brain of Pekin ducks, and even though IL-1β seems equally pyrogenic, the action of IL-1β is dependent on IL-6, since anti-IL-6 abolished an IL-1β-induced fever.

Until now, the involvement of IL-6 in the avian febrile mechanism has been limited to studies investigating the regulation of circulating IL-6 after immune challenge. From the results of these studies, it is evident that circulating avian immune cells secrete IL-6 upon immune challenge (9, 47); yet neither the physiological course of IL-6 in birds nor the relationship between IL-6 and febrile mediation has been elucidated. The results of DeBoever et al. (9) and Nakamura et al. (27) suggest that peripheral IL-6 contributes to febrile mediation, because the peak concentrations of plasma IL-6 occurred during the rising phase of the fever response in chickens. Our results suggest that circulating IL-6 either crosses the BBB or instigates the de novo synthesis of brain IL-6 to mediate fever in birds.

Effect of Systemic Diclofenac on Centrally Induced IL-1β Fever

Systemic treatment with a PG synthesis inhibitor, diclofenac, abolished the fever induced by an intracerebroventricular injection of IL-1β in our birds, indicating that PGs may well fulfill a role in the central component of the febrile mechanism in Pekin ducks. This result, together with the finding that central IL-1β and IL-6 are pyrogenic in birds, suggests the following sequence of events within the central component of the avian febrile mechanism: IL-1β gives rise to bioactive IL-6, which stimulates an accelerated synthesis of PGs. The scenario from this point is unknown but may be similar to that in mammals, where these PGs then adjust the sensitivity of warm-sensitive neurons in the avian brain stem to mediate fever. Alternatively, IL-1β may give rise to bioactive IL-6, and IL-6 may serve as a terminal mediator of fever in Pekin ducks.

Our first scenario is supported by evidence that 1) innate immune activation triggers the release of IL-6 in birds (9, 27), 2) avian thrombocytes, a major component of the innate immune system in birds, upregulate their expression of IL-6 mRNA, as well as the important PG-synthesizing enzyme COX-2, after immune challenge (41), and 3) chickens develop fever following hypothalamic injection of PGE2 (1). There is convincing evidence that IL-6 and PGE2 are critically important for febrile mediation in mammals (5, 29, 35), even though the mechanism by which IL-6 instigates changes in the central nervous system to cause fever in mammals is still controversial. Fever is an evolutionarily conserved response, and this scenario supports the notion that the crucial role of IL-6 and PGs in febrile mediation has also been evolutionarily retained.

Our alternative scenario follows from the absence of direct evidence that links central endogenous PGs to fever in birds. Although intracerebroventricular injection of PGE1 and PGE2 causes fever in chickens (1, 2, 30) and PG synthesis inhibitors have been shown to modulate the fever response in chickens and ducks (2, 14, 23), no one has demonstrated upregulated levels of PGs in the brain of febrile birds. In the present study, we abolished IL-1β-induced fever with diclofenac, but we did not find PGE2 to be upregulated in the CSF of febrile ducks, even though we measured PGE2 concentrations at a time that coincided with Tb values nearing the peak of the febrile response. Similarly, Fraifeld et al. (13) did not find higher-than-normal levels of PGE2 in the brain of febrile chickens.

The nonsteroidal anti-inflammatory drug (NSAID) diclofenac is a nonselective inhibitor of PG synthesis. If PGs are involved in febrile mediation in birds, it is possible that another class of PG serves as the final mediator of fever. However, this notion contradicts the results of Nomoto (32), who found that a different NSAID, indomethacin, had no effect on the fever response.
response in pigeons when injected centrally, thereby excluding PGs as potential terminal mediators of the febrile response in pigeons. In contrast to these results in pigeons, evidence that indomethacin abolished LPS-induced fever in chickens (23) and that diclofenac reduced LPS-induced fever in ducks (14) indicates that PGs may be more important for febrile mediation in some avian species than others. Alternatively, differences in the pharmacological action of indomethacin and diclofenac might account for the differential effect of these PG synthesis inhibitors on the biological pathway that leads to febrile mediation in birds. Diclofenac was shown to be more potent than indomethacin at inhibiting TNF-induced NF-κB activation (50). NF-κB is known to regulate genes that mediate inflammation, including COX-2, as well as proinflammatory cytokines. It follows then that, in our ducks, diclofenac could have inhibited IL-1β-induced fever via its inhibitory actions on the synthesis of proinflammatory cytokines, in particular IL-6. This scenario supports the idea that fever in birds might be mediated independent of PGs and that NSAIDs exert their action on the avian febrile mechanism by inhibiting alternative mediators of the febrile response to that observed in mammals.

Absence of PGE2 in CSF of LPS-Treated Pekin Ducks

Fever in Pekin ducks, in response to systemic LPS treatment, comprises a monophasic rise in Tb, starting at ~90 min posttreatment and reaching peak values (on average 1.5°C higher than normal Tb) at ~180 min posttreatment (15, 24). This rise in Tb is metabolically costly; however, once the peak Tb has been reached, metabolic energy utilization returns to baseline levels (25), indicating that pyrogens exert their action on thermoregulatory control during the rising phase of the febrile response. For this reason, we chose to measure PGE2 concentrations in the CSF of Pekin ducks 150 min after LPS treatment, during the rising phase, but before the peak of fever. Previous studies with birds confirmed that systemic LPS treatment initiates an increase in plasma IL-6 concentrations (9, 27), and there is evidence that, in pigeons, LPS stimulates peripheral PG synthesis (32).

In mammals, circulating IL-6 is known to participate in febrile mediation following LPS treatment (5, 6, 35), and recent evidence suggests that IL-6 facilitates the entry of PGs into the brain (29). If we considering the evidence that systemic treatment with diclofenac reduced the LPS-induced fever in Pekin ducks, it is alluring to deem PGs a mediator in the avian febrile response. If in birds, like mammals, IL-6 aids the transfer of peripherally derived PGs into the brain, then diclofenac very likely moderates LPS-induced fevers in Pekin ducks (14) by inhibiting the synthesis of circulating PGs and, thus, the amount of PG available to enter the brain and also by allowing those peripherally derived PGs to enter the brain. However, we did not find higher-than-baseline concentrations of PGE2 in the CSF of ducks made febrile with LPS at a time when febrile mediation should have been maximal, corroborating the findings of a study in chickens that measured no change in PGE2 during fever (13).

It is also possible that the downregulation of IL-6 alone could account for the moderation of LPS-induced fever in ducks. In addition to abetting the transfer of peripherally derived PGs into the brain, IL-6 might affect the controllers of thermoregulation by 1) crossing the BBB, 2) instigating the de novo synthesis of IL-6 in the avian brain, and 3) serving as an activator of febrile mediators, other than PGE2, in the brain. In mammals, IL-6 was shown to act on the brain sites that lack a BBB (37). Rummel et al (39) and Harre et al (17) showed that, in the presence of circulating IL-6, nuclear translocation of the signal transducer and activator of transcription 3 molecule was induced in cells of the organum vasculosum lamina terminalis and subfornical organ, and this likely activated the PG-synthesizing pathway. Because of the limited knowledge available regarding the avian febrile mechanism, we can only speculate on the manner by which IL-6 induced fever in our ducks. Nevertheless, it is clear from the results of our study and from the results of mammalian studies that IL-6 and PGs are not exclusively responsible for LPS-induced fevers. Macrophage inflammatory protein-1β, corticotropin-releasing factor, and endothelin-1 have been identified as PG-independent mediators of fever in mammals (11, 12). In addition, Fraga et al (12) showed that, in mammals, LPS and endogenous pyrogens (except IL-1β and PGs) recruit the opioid system to cause a μ-receptor-mediated fever independent of PG. In mammals, the opioidergic system seems to be activated simultaneously with, or after, PG synthesis (12).

Although nothing is known about the contribution of macrophage inflammatory protein-1β, corticotropin-releasing factor, endothelin-1, or endogenous opioids to avian fever, it is likely that the avian febrile mechanism comprises diverse biological pathways, and it is plausible that the importance of these pathways differs between avian species.

In conclusion, we show that avian IL-1β and avian IL-6 are pyrogenic within the avian brain. These proinflammatory cytokines may well mediate fever by instigating an accelerated synthesis of brain-derived PG. However, PGE2 does not seem to be involved in febrile mediation in birds, at least not during the latter part of the rising phase of the febrile response in Pekin ducks. In mammals and in birds, LPS seems to employ various biological pathways to mediate fever.

Perspectives and Significance

Although fever is considered an evolutionarily conserved immunological response to invading pathogens, the results of this study show clear differences in the manner in which the proinflammatory cytokines IL-1β and IL-6 initiate fever in the avian and mammalian brain. In Pekin ducks, the pyrogenicity of brain IL-1β is dependent on the availability of brain IL-6. Furthermore, even though PGs have a central role in the mammalian febrile mechanism, we and others have presented results that call into question the significance of PGE2 in the avian febrile mechanism because of a lack of direct evidence for the upregulation of this prostanoid in febrile birds.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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