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Gemfibrozil disrupts the metabolism of circulating lipids in bobwhite quails

Sophie Bussière-Côté [a], Teye Omlin [a], Eliana de Càssia Pinheiro [b], Jean-Michel Weber [a,*]

[a] Biology Department, University of Ottawa, 30 Marie Curie, Ottawa, ON K1N 6N5, Canada
[b] Department of Physiological Sciences, University of Brasília, Brasília, DF, Brazil

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ABSTRACT

The circulating lipids of birds play essential roles for egg production and as an energy source for flight and thermogenesis. How lipid-lowering pharmaceuticals geared to prevent heart disease in humans and that are routinely released in the environment affect their metabolism is unknown. This study assesses the impact of the popular drug gemfibrozil (GEM) on the plasma phospholipids (PL), neutral lipids (NL), and nonesterified fatty acids (NEFA) of bobwhite quails (*Colinus virginianus*). Results show that bird lipoproteins are rapidly altered by GEM, even at environmentally-relevant doses. After 4 days of exposure, pharmacological amounts cause an 83% increase in circulating PL levels, a major decrease in average lipoprotein size measured as a 56% drop in the NL/PL ratio, and important changes in the fatty acid composition of PL and NEFA (increases in fatty acid unsaturation). The levels of PL carrying all individual fatty acids except arachidonate are strongly stimulated. The large decrease in bird lipoprotein size may reflect the effects seen in humans: lowering of LDL that can cause atherosclerosis and stimulation of HDL that promote cholesterol disposal. Lower (environmental) doses of GEM cause a reduction of %palmitate in all the plasma lipid fractions of quails, but particularly in the core triacylglycerol of lipoproteins (NL). No changes in mRNA levels of bird peroxisome proliferator-activated receptor (PPAR) could be demonstrated. The disrupting effects of GEM on circulating lipids reported here suggest that the pervasive presence of this drug in the environment could jeopardize reproduction and migratory behaviours in wild birds.

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1. Introduction

Birds use lipoproteins to transport most of the energy necessary for flight (Jenni-Eiermann and Jenni, 1992; Guglielmo et al., 2002; Weber, 2009), thermogenesis (Vaillancourt et al., 2005; Vaillancourt and Weber, 2007), and egg production (Walzem et al., 1999; Salvante et al., 2007). They support these essential functions by maintaining high lipoprotein levels (~3 times mammalian values) that account for >85% of total plasma lipids (Jenni-Eiermann and Jenni, 1992; Guglielmo et al., 2002; Vaillancourt and Weber, 2007; Alvarenga et al., 2011). The popular fibrate drug gemfibrozil (GEM) is constantly released from sewage treatment plants into surface waters around the world, where it is found in ng/L to μg/L concentrations (Daughton and Ternes, 1999; Metcalfe et al., 2003; Paxeus, 2004). To aggravate the problem, GEM accumulates in fish with a bioconcentration factor of 65 to 113 and it shows great potential for biomagnification (Mimeault et al., 2005; Brown et al., 2007). This drug belongs to a class of lipid-regulating pharmaceuticals geared to alter the lipoprotein profile of humans treated for coronary artery disease. It acts by binding to the transcriptional regulator, peroxisome proliferator-activated receptor α (PPARα) (Forman et al., 1997) that controls the expression of genes regulating major aspects of lipid metabolism (Mandard et al., 2004). In humans, GEM reduces low-density lipoprotein levels (LDL: a large lipoprotein that carries cholesterol to the vasculature where it can cause lesions) and it increases high density lipoproteins (HDL: a smaller lipoprotein that carries cholesterol to the liver for degradation) (Smith et al., 2002; Nyalala et al., 2008). Circulating lipids include non-esterified fatty acids (NEFA), as well as neutral lipids (NL) and phospholipids (PL): the two main components of lipoproteins. Because NL form the core and PL the surface, the NL/PL ratio increases with lipoprotein size and decreases with lipoprotein density (NL being lighter than PL and surface proteins). No study has assessed how bird lipoproteins respond to environmental contaminants, especially those designed to manipulate lipid metabolism in humans. For example, piscivorous birds have become a significant concern because they are eventually exposed to higher GEM levels than found in the fish they eat. In this study, we have started to investigate how fibrate drugs affect circulating lipids in birds, using bobwhite quail (*Colinus virginianus*) as a convenient model easily kept in captivity. The goal of this study was to quantify the impact of GEM on circulating phospholipids, neutral lipids, and nonesterified fatty acids. More specifically, we wanted to determine the effects of pharmacological and

* Corresponding author. Tel.: +1 613 562 5800x6007; fax: +1 613 562 5486.
E-mail address: jmweber@uOttawa.ca (J.-M. Weber).

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environmental doses of the drug on the NL/PL ratio and the fatty acid composition of NL, PL and NEFA. To examine potential mechanisms of action in birds, the effects of GEM on the expression of PPAR genes were also assessed in the pectoral muscle and liver. The conserved nature of the lipid transport system among vertebrates (Havel, 1987; Tocher, 2003) suggests that birds should respond to fibrate drugs similarly to humans. Therefore, we hypothesized that GEM would reduce the average size of quail lipoproteins (thereby lowering the NL/PL ratio), as well as the concentration of total plasma lipids, possibly targeting key fatty acids. We also anticipated that the drug would increase the expression of PPARs, particularly PPARα.

2. Methods

2.1. Animals

Tamed adult bobwhite quails of both sexes (*C. virginianus*; 235 ± 10 g; N = 24) were obtained from a local supplier (St. Hyacinthe, Québec, Canada). The animals were handled daily from hatching to familiarize them with humans and minimize future experimental stress. The quails were fed a commercial starter turkey diet (21% protein, 3.5% lipids, 4% fibre; LaBonté Belhumeur, St. Bonaventure, Québec, Canada) and had access to water ad libitum. They were identified individually by leg banding, kept indoor in a windowless room (4 × 2 × 2 m) on a substrate of wood chips (22 °C; 50% humidity; 12 h:12 h light:dark photoperiod), and were acclimated to these conditions for 3 weeks before experiments.

2.2. Gemfibrozil treatment and sample collection

In addition to their regular diet, the animals received daily 0.7 ml doses of oil by gavage over 4 days. A 14 G stainless steel gavage needle was used to administer the oil and the procedure lasted <30 s per animal each day. The birds were randomly assigned to 3 treatment groups: C (controls: corn oil only), LG (low dose gemfibrozil: 5 mg/day in corn oil), and HG (high dose gemfibrozil: 35 mg/day in corn oil). The amount of gemfibrozil given to the HG group was calculated to simulate the recommended dose for humans (1200 mg/day) (Smith et al., 2002) after scaling for resting metabolic rate (RMR of 250 g-bird/RMR of 70 kg-human = 0.0292). At the end of the 4-day treatment, the birds were anesthetized with isoflurane and euthanized by cervical dislocation. Blood samples were collected by cardiac puncture (~2 mL) using EDTA as anticoagulant. Plasma was immediately separated by centrifugation (10 min at 12,000 g). Pectoral muscle and liver samples (~1 g each) were freeze-clamped in liquid nitrogen within 3 min after death. Plasma and tissue samples were stored at −80 °C until analyzed.

2.3. Lipid analysis

Plasma lipids were extracted with chloroform:methanol (2:1 v/v) (Folch et al., 1957) and centrifuged (10 min at 2000 g). Pellets were discarded and the supernatants were filtered before adding 0.25% KCl to help eliminate water-soluble compounds. After shaking, the mixture was placed in a 70 °C water bath to separate aqueous and organic phases. The organic phase was dried under N_2 and total lipids were resuspended in chloroform. Neutral lipids (NL), nonesterified fatty acids (NEFA), and phospholipids (PL) were separated by sequential elution from Supelclean solid-phase extraction columns (1 mL LC-NH_2; Sigma, St. Louis, MO, USA). NL were eluted with chloroform:isopropanol (2:1 v/v), NEFA with isopropyl ether:acetic acid (98:2 v/v), and PL with methanol (Maillet and Weber, 2006). The fatty acid composition of the 3 lipid classes was measured by gas chromatography following methylation for NEFA or acid transesterification for PL and NL (Chapelle and Zwingelstein, 1984; Abdul-Malak et al., 1989). Heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were analyzed on an Agilent Technologies 6890 N gas chromatograph equipped with a fused silica capillary column (Supelco DB-23, 60 m × 0.25 mm i.d., 0.2 μm film thickness) using hydrogen as carrier gas. Detailed conditions for gas chromatography analysis were described previously (Magnoni and Weber, 2007). Individual fatty acids were identified by determining exact retention times with authentic standards (Sigma-Aldrich, St. Louis, MO, USA). Only the fatty acids accounting for >1% of total fatty acids in each lipid fraction are reported as individual fatty acids. However, even minor fatty acids (when detected at <1% of total) are accounted for in reported values for total saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA).

2.4. PPAR expression

2.4.1. RNA extraction and cDNA synthesis

The expression of PPAR α, β and γ transcripts was measured in the pectoral muscle and liver. All reagents were obtained from Invitrogen (Burlington, ON, Canada) unless indicated otherwise. Total RNA was isolated from ~0.1 g frozen tissue using TRIzol® reagent (Gibco BRL, Burlington, ON, Canada). Isopropanol and linear acrylamide (Ambion, Austin, TX, USA) were added to aid RNA precipitation. The samples were placed for 30 min on dry ice and centrifuged to collect RNA pellets (5 min; 12,000 g; 4 °C). The RNA concentration and quality were verified using NanoDrop 1000 (Thermo Fisher Scientific). The RNA was DNase-treated (DNase I, Amplification Grade) before cDNA synthesis to avoid degradation. The reaction mixture contained 1 μg of total RNA, 200 ng random hexamers, and RNase-/DNase-free water for a total volume of 20 μL. The mixture was incubated at 65 °C for 10 min, quickly chilled on ice, and briefly spun at 13,750 g. Four μL 5× reaction buffer, 2 μL 0.1 M DTT, 1 μL 10 mM dNTPs, and 1 μL RNase inhibitor were added, gently mixed, and the solution was pre-incubated at 37 °C for 2 min. One μL M-MLV Reverse Transcriptase or 1 μL of water (NoRT) was added and the reaction was allowed to proceed for 60 min at 37 °C. The reaction was inactivated at 70 °C for 15 min and stored at −20 °C.

2.4.2. Q-PCR

Primers for PPAR α, β, and γ of bobwhite quail were as described in Nagahuedi et al. (2009). PPAR mRNA levels in samples from control and gemfibrozil-treated animals were quantified by real-time RT-PCR using Brilliant SYBR Green (Stratagene, Cedar Creek, TX, USA) with a 100 nM ROX reference dye. The reaction was performed on a MX3000® Multiplex Quantitative PCR system (Stratagene, Mississauga, ON, Canada) in a total volume of 25 μL. Each PCR reaction contained the following: 20 ng first-strand cDNA template, 1× QPCR buffer, 5 mM $MgCl_2$, 150–600 nM gene specific primer, 0.5× SYBR green (Invitrogen), 200 μM dNTPs, 1.25 U HotStarTaq (Invitrogen), and 20 μM ROX reference dye. Thermal cycles for PPAR α were: 95 °C for 15 min, followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a detection step at 80 °C for 18 s. For PPAR β and γ, thermal cycles were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 54 s, and a detection step at 80 °C for 22 s. Real-time PCR data were analyzed using the MxPro Software Package (Stratagene). We were unable to find adequate housekeeping genes to normalize PPAR expression (for example, the expression of 18S decreased by 42% with exposure to the high dose of GEM). Under these conditions, we have normalized PPAR expression to total RNA because it provides the least biased estimates (Bustin, 2000, 2002).

2.5. Statistical analyses

Statistical differences were assessed using one-way analysis of variance (ANOVA). When significant changes were detected, the Bonferroni method was used for pairwise comparisons. When the assumption of normality was not met, a Kruskal–Wallis ANOVA on ranks was used with the Dunn's method for pairwise comparisons. Percentages were

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