Fatty acid binding protein in heart and skeletal muscles of the migratory barnacle goose throughout development

MAURICE M. A. L. PELSERS, PATRICK J. BUTLER, CHARLES M. BISHOP, AND JAN F. C. GLATZ

The long-distance migratory flights of birds are extremely arduous and potentially fatal, especially for the juveniles that are hatched during the summer and must develop sufficient endurance to migrate a few months later in the autumn. An example of one such avian migration is exhibited by the Svalbard popula-

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tion of the barnacle goose (Branta leucopsis), which flies ~2,500 km from its Arctic breeding grounds to overwinter in southwestern Scotland when the goslings are ~12 wk of age (5–7). Thus the aims of this study were to isolate and characterize FABP from barnacle goose muscles with different fatty acid oxidation capacities (heart, pectoralis, and semimembranosus) and to determine the degree of correlation between the FABP content of these various locomotor muscles in developing barnacle goose and their relative stages of functional and structural development. Differences in FABP content could therefore provide a useful insight into the avian metabolic mechanisms underpinning the ability to perform long-distance flights. The pectoralis was divided into peripheral and deep fibers, because it is known that this mixed-type muscle contains a higher percentage of oxidative fibers in the deeper layers than in the peripheral layers (23). The FABP content of the pectoralis muscles of postfledging birds was also compared between samples obtained from wild and captive populations of geese to assess the relative importance of flight activity on the expression of muscle FABP.

MATERIALS AND METHODS

Capture of birds. Captive barnacle geese were kept in outdoor aviaries at Birmingham, UK (between 1992 and 1995). Goslings were sampled at 3 (n = 4), 5 (n = 8), 6 (n = 4), 7 (n = 10), and 20 wk (n = 2) of age. Captive adult birds (n = 6) that were >2 yr old and flight restricted so that they could only fly for a few meters were sampled 12 wk after the average hatch date for the population of captive goslings. Wild barnacle geese were caught using corral nets or small pneumatic-powered cannon nets and sampled between 1991 and 1995 in two areas on the western coast of the island of Spitsbergen, Svalbard (7). Samples were taken from wild goslings at 7 (n = 4) and 11.5 wk posthatch (n = 2) and from wild adult geese at 7 (n = 8), 9 (n = 3), and 10.5 wk (n = 9) after the average hatch date for the population of wild goslings.

Tissue sampling. After terminal anesthesia of the bird (injection of 200 mg/kg pentobarbital sodium ip or iv or inhalation of 5% halothane), the skin overlaying the pectoralis major muscle was quickly dissected away and the muscle was exposed. Peripheral and deep biopsies 0.5 cm wide and 1 cm long were taken 20% of the way along the breast muscle from the anterior end and 1–2 cm lateral to the sternum but parallel to the direction of the muscle fibers (10). Small amounts of tissue (100–200 mg) were placed in preweighed polypropylene tubes, weighed again, and stored in liquid nitrogen (−196°C) until analysis. In addition, samples were taken from the left ventricular wall of the heart and finally by sonicating (4×15 s, MSE ultrasonic disintegrator). Aliquots of 100 and 500 µl homogenate were stored at −80°C. Before FABP measurement, samples were thawed and centrifuged for 10 min at 15,000 g and 4°C. The supernatant was then used for FABP measurements.

Purification of FABP from goose heart. To quantify the percentage of cross-reactivity between human and goose FABP, purification of FABP from goose heart was performed essentially according to Van Nieuwenhoven et al. (30), but the S200 gel filtration step was omitted because of the small amount of protein available. In brief, heart homogenates were pooled and centrifuged at 15,000 g. The supernatant was then applied to an anion exchange column (Sepharose Q) equilibrated with 10 mM imidazole buffer at pH 7.0. After they were washed with equilibration buffer (100 ml), sample fractions (6 ml) were eluted with a gradient of 0–30 mM NaCl in 10 mM imidazole at pH 7.0. Each eluted fraction was checked for fatty acid binding capacity with the Lipidex assay (12). FABP was also measured with an ELISA for human H-FABP (34). After pooling the fractions containing FABP and dialyzing them with phosphate buffer, we performed a gel filtration step (S-75). The samples were again checked for fatty acid binding capacity with the Lipidex assay (12), and the purity of the eluted proteins was assessed by SDS-PAGE.

Protein determination. Total tissue protein content was determined in the muscle homogenates by use of a microcinchoninic acid kit (protein assay no. 23235; Pierce, Rockford, IL), using BSA as the standard (27). The protein concentration of purified goose H-FABP was also determined with this kit, as well as via optical density at 280 nm using the extinction coefficient found for both human and bovine H-FABP [E280 (1 mg/ml) = 0.87 and 0.85, respectively] (26).

Electrophoresis and Western blotting. Western blotting experiments were performed with a Mini-Protein II system (Bio-Rad, Hercules, CA) to study the ability of existing mouse monoclonal antibodies (MAbs) directed against human H-FABP to detect FABP in goose muscle. For this, muscle homogenates were diluted 1:4 in a sample buffer containing 2%(wt/vol) SDS and 5%(wt/vol) β-mercaptoethanol and then incubated for 10 min at 95°C. Separation of the proteins took place in parallel in two identical 15% polyacrylamide Tris-glycine gels (1 h, 200 V). Thereafter, the proteins of gel A were stained with Coomassie brilliant blue. The proteins of gel B were blotted (1 h, 100 V) on a 0.45-µm nitrocellulose sheet. After blocking with 1%(wt/vol) nonfat dry milk, the blot was incubated overnight at 4°C with a mouse MAb (66E2) directed against human H-FABP (23) and of eluted chromatography fractions were measured with the Lipidex assay (34). In brief, mouse MAb 67D3 directed against human H-FABP was coated as the catcher antibody overnight at 4°C on a Falcon 3912 microtiter plate (PVC; Becton Dickinson, Oxnard, CA) in 0.1 M carbonate buffer at pH 9.6. After washing with PBT buffer [0.01 M phosphate, pH 7.2, containing 0.1%(wt/vol) BSA (Sigma A-4503) and 0.05%(vol/vol) Tween 20], diluted muscle homogenates (1/1,000) and MAb 66E2 (labeled with horseradish peroxidase) as the detector antibody were added, followed by incubation for 30 min at 37°C. Thereafter, the plates were washed with PBT buffer. 3,.3’,5,5’-Tetramethylbenzidine was added as a substrate, and after 7 min of colorization, the reaction was stopped by rinsing the blot with distilled water.

Immunoassay of FABP. FABP contents of tissue samples and of eluted chromatography fractions were measured with a direct noncompetitive ELISA (sandwich ELISA) of the antigen capture type using recombinant H-FABP as a standard (34). In brief, mouse MAb 67D3 directed against human H-FABP (23) was coated as the catcher antibody overnight at 4°C on a Falcon 3912 microtiter plate (PVC; Becton Dickinson, Oxnard, CA) in 0.1 M carbonate buffer at pH 9.6. After washing with PBT buffer [0.01 M phosphate, pH 7.2, containing 0.1%(wt/vol) BSA (Sigma A-4503) and 0.05%(vol/vol) Tween 20], diluted muscle homogenates (1/1,000) and MAb 66E2 (labeled with horseradish peroxidase) as the detector antibody were added, followed by incubation for 30 min at 37°C. Thereafter, the plates were washed with PBT buffer. 3,.3’,5,5’-Tetramethylbenzidine was added as a substrate, and after 7 min of colorization, the reaction was stopped with 2 M H2SO4. The spectral absorption was measured at 450 nm with a Titertek Multiscan II spectrophotometer (Labsystems, Helsinki, Finland).

Statistics. All values are expressed as means ± SE for the indicated number of samples. Statistical comparisons be-
between different age groups within a given population were analyzed using one-way ANOVA, followed by a Fisher's protected least significant difference post hoc test (Statview 4.0 software) to identify which age groups were significantly different from each other at the 5% level. Comparisons between specific age groups from the two populations were performed using Student's two-tailed t-test, with significance at the 5% level.

RESULTS

Isolation and characterization of FABP from goose heart. FABP was purified from adult goose heart by two chromatographic steps. Anion exchange chromatography yielded two sets of fractions (Fig. 1A, fractions 16-24 and 25-30) that showed appreciable noncovalent binding of long-chain fatty acids as assessed by the Lipidex assay and also showed immunoreactivity with antibodies directed against human H-FABP. These two peaks most likely reflect FABP isoforms differing in isoelectric point, as also occur in heart tissue from other species (11). Subsequently, the combined FABP-containing fractions were subjected to gel filtration chromatography to yield one major peak of proteins (fractions 69-79) that also showed fatty acid binding (Fig. 1B).

In view of the appreciable sequence homology between H-FABPs from different species (11), we explored the possibility of using the available mouse MAbs directed against human H-FABP in a heterologous assay for goose FABP. We earlier found that human H-FABP MAbs cross-react with mouse, rat, dog, and bovine H-FABP to an appreciable extent (26–60%) (26). Comparison of purified goose H-FABP in a calibration curve for human H-FABP revealed an overall cross-reactivity of 66% (Fig. 2), indicating that it is acceptable to use this immunoassay to quantify FABP in goose muscle samples.

A Western blot was conducted to check if the MAbs used in the human H-FABP ELISA recognized exclusively the 15-kDa goose H-FABP. Figure 3 shows that the mouse MAbs recognize a single 15-kDa protein in pectoralis (lanes 2 and 3), heart (lane 4), and semimembranosus leg muscle (lane 5), the molecular mass of which matches that of purified goose H-FABP. Thus goose FABP can be quantitatively determined in these muscle samples using the established heterologous assay for human H-FABP either by using goose FABP as standard or by allowing for the 66% cross-reactivity of the mouse MAbs.

The Western blot analysis indicated marked differences in FABP content among these goose muscles, and such differences were confirmed by subsequent ELISA measurements (see below).

FABP content of goose muscle samples during development. Figure 4 shows the FABP content (mean ± SE) of semimembranosus leg muscle and cardiac muscle during the development of captive goslings between 3 and 7 wk of age and compares these results to samples taken from captive and wild adult geese. The semimembranosus muscle contains relatively low quantities of FABP (8.4 ± 1.9 µg/g wet wt, n = 43) compared with those of the cardiac muscle (62.6 ± 10.6 µg/g wet wt, n = 43). However, there is no significant change in

![Fig. 1. A: anion exchange chromatography on a Sepharose Q fast-flow column (1.6 × 15 cm) of cytosolic protein fraction of goose heart homogenate. Heart-type fatty acid binding protein (FABP) was eluted with a linear gradient of NaCl (bold solid line) in starting buffer. Elution was monitored at A280 nm (light solid line) for protein. Fatty acid binding activity was measured with the Lipidex assay and FABP antigenic activity with an ELISA using antibodies against human heart-type FABP (see MATERIALS AND METHODS), yielding similar results; only the latter is shown (dashed line; signal is expressed as A450 nm). B: gel permeation chromatography on a Superdex-75 column (2.6 × 60 cm) with pooled fractions 15-30 of anion exchange run (A). Elution was monitored at A280 nm (solid line). Fatty acid binding capacity was measured with Lipidex assay using [14C]oleate (dashed line).](http://ajpregu.physiology.org/)

![Fig. 2. ELISA with a calibration curve of human heart-type FABP (●) and goose heart-type FABP (○).](http://ajpregu.physiology.org/)
FABP content throughout the development of the goslings or with respect to captive and wild adult geese in either cardiac (ANOVA: $F = 1.1$, $P = 0.39$, $n = 43$) or semimembranosus muscles (ANOVA: $F = 1.08$, $P = 0.40$, $n = 43$). During development, significant differences in FABP content were seen in the pectoralis flight muscle of both the deep (ANOVA: $F = 29.3$, $P < 0.0001$, $n = 47$) and peripheral (ANOVA: $F = 48.6$, $P < 0.0001$, $n = 60$) sites (Fig. 5). In the peripheral fibers, FABP content was $2.6 \pm 0.2 \mu g/g$ wet wt ($n = 4$) in 3-wk-old goslings and $26.0 \pm 3.2 \mu g/g$ wet wt ($n = 10$) in fledging goslings at 7 wk of age, whereas in the deep fibers, FABP was $4.2 \pm 1.0$ ($n = 4$) and $42.6 \pm 6.1 \mu g/g$ wet wt ($n = 8$) in 3-wk-old and fledging goslings, respectively. FABP contents of captive fledging goslings in both peripheral and deep sites were not significantly different from those from either captive goslings at 20 wk of age or from long-term captive adult birds ($25.2 \pm 3.7$ and $59.7 \pm 8.3 \mu g/g$ wet wt for peripheral and deep sites, respectively; Fig. 5). Values in wild fledging goslings were not significantly different from their captive counterparts ($30.5 \pm 2.7$ and $32.9 \pm 4.0 \mu g/g$ wet wt, respectively) but were substantially lower than those obtained for two wild goslings at 11.5 wk of age and significantly lower ($P < 0.005$) than wild, premigratory adult geese around 10.5 wk after the mean hatch date of the population ($60.5 \pm 3.6$ and $94.3 \pm 3.6 \mu g/g$ wet wt, respectively; Fig. 5, C and D). Values from the wild, premigratory geese were also significantly higher than those from wild adult geese 7 wk after the mean hatch date of the population ($P < 0.02$) and those from captive adult geese ($P < 0.04$). However, values from captive adults and wild adults at 7 wk posthatch were not significantly different for samples taken from the deep site, but values from the peripheral site were significantly lower ($P < 0.02$) in the captive adult geese. For almost every single age group, in both wild and captive birds, there is a significantly higher content of FABP in the deep muscle layers compared with those of the peripheral samples.

Muscle total protein content. Although the total protein content of the muscle samples showed marked variation in heart (149–274 mg/g wet wt), pectoralis (98–343 mg/g wet wt), and semimembranosus muscles (222–333 mg/g wet wt), in none of the muscles was a significant relation between tissue protein content and development observed (data not shown). Thus FABP

![Fig. 3. SDS-PAGE (left) and Western blot (right) of different goose muscle homogenates. Lane 1, purified goose heart FABP; lane 2, adult pectoralis; lane 3, pectoralis of 5-wk-old goose; lane 4, heart of 5-wk-old goose; lane 5, adult semimembranosus leg muscle; lane 6, molecular mass markers. All lanes contain 10 µl of 4% homogenate.](image)

![Fig. 4. FABP contents of heart muscle (open bars) and semimembranosus leg muscle (solid bars) at various stages during development in captive goslings (A) and postbreeding captive and wild adults (B and C, respectively). Captive adults had been flight restricted for at least 2 yr. Note that migration takes place ~12 wk after mean population hatch date of goslings. Data are given as means ± SE, with numbers over bars representing numbers of samples studied. ww, Wet weight.](image)
content of the muscle samples expressed per milligrams total protein showed similar developmental changes as those expressed per grams wet weight of tissue (data not shown).

**DISCUSSION**

Using the purification protocol for human H-FABP, we were able to isolate FABP from goose heart muscle. This protein showed an ability to bind fatty acids and, in comparison with human H-FABP, a cross-reactivity of 66% with mouse anti-human H-FABP MAbs. The ability to detect FABP using MAbs directed against human H-FABP in various muscle tissues taken from barnacle geese supports the hypothesis that there are general epitopes for the FABPs in heart and skeletal muscles taken from widely different species (and even different classes of vertebrates). This is consistent with earlier results on the assay of H-FABP in rat, mouse, and dog tissues (A. H. Kleine, Y. F. de Jong, and J. F. C. Glatz, unpublished observations).

This is the first time that FABP has been quantified in the locomotor muscles of a bird that is capable of migrating long distances. In general, the FABP content in the adult barnacle goose skeletal muscles (pectoralis and semimembranosus) were similar (10–100 µg/g wet wt) to those found in the skeletal muscles of higher vertebrates such as the rat (10–300 µg/g wet wt) (32) and in humans (10–160 µg/g wet wt) (29). However, the FABP content of the cardiac muscle of barnacle geese was markedly lower (60 µg/g wet wt) than that of rats (700 µg/g wet wt) (32) or humans (500 µg/g wet wt) (11), and these differences were not related to differences in tissue protein content. A preliminary investigation by the authors of the present study of relative FABP content in the heart and pectoralis muscles of other avian species (sparrow, starling, and crow) indicated that these species may have higher FABP contents in their cardiac and flight muscles than those found in the barnacle goose but that the ratio of heart to pectoralis FABP content (sparrow 2.1, starling 0.7, crow 0.7) was similar to that of barnacle geese (0.9). These differences were also seen in the Western blot analysis (data not shown). Thus, assuming that FABP content is a reliable indicator of fatty acid utilization, it would appear that FABP-mediated fatty acid transport is lower in barnacle goose heart than in human or rat heart, although the physical demands for migrating geese are at least as high as those of a running mammal.

A source of energy in addition to that of fatty acids could be provided by metabolizing ketone bodies produced from fatty acids in the liver. Certainly, the maximum activities of the enzymes involved in ketone utilization (3-hydroxybutyrate dehydrogenase, 3-ketoacid CoA-transferase, and acetoacetyl-CoA thiolase) are much higher in the cardiac muscle compared with those in the pectoralis muscle of birds (3) and may indicate that the avian heart is relatively well adapted to utilize ketone bodies. However, the mammalian heart also has relatively high levels of activity for enzymes involved in ketone utilization compared with those of the leg muscles (3). In contrast, FABP content in the flight muscles of locusts shows very high FABP values (1,300 µg/g wet wt) (14) but very low ketone body activities (3), and this is consistent with a high demand for fatty acid catabolism (14). Unfortunately, values for locust cardiac muscle were not presented in these studies. Thus results from the limited number of studies available suggest that there may be an inverse relationship between very high tissue FABP content and very high ketone body catabolism, at least in the skeletal muscles.

The FABP contents of the heart and pectoralis muscles of adult barnacle geese were found to be of similar magnitude (60–80 µg/g wet wt). This result agrees with the similar maximum activities of citrate synthase (CS), a general indicator of tissue aerobic capacity,
found in these two tissues (5). However, maximum activity of 3β-hydroxacyl-CoA dehydrogenase (HAD), a specific indicator of fatty acid oxidative capacity, was found to be ~50% higher in the heart than in the pectoralis muscle of barnacle geese (5). Thus it is clear that both these muscles are capable of oxidizing fatty acids at a relatively high rate compared with the leg muscles and both HAD and FABP are expressed at their maximum concentrations in the flight muscles of the physically mature geese immediately before the onset of the autumn migration. In addition, the FABP content and the activity of CS (10) measured in the peripheral part of the pectoralis muscle are consistently lower than those found in the deeper sites. This confirms that the deeper sites in the pectoralis muscle consist of fibers with a relatively higher oxidative capacity (10, 24) and a greater specialization for β-oxidation of fatty acids. The same difference is seen in rats in which there is a >10-fold difference in FABP content between superficial and deep layers of rat quadriceps muscle (32).

The pectoralis muscle shows an enormous increase in FABP content throughout development from 3 wk of age up to the migration (peripheral samples show a 10-fold difference and deep samples a 25-fold difference). This result is also similar to the results for the maximum activities of both CS and HAD in developing wild goslings (5). In contrast, the semimembranosus muscle of the leg shows relatively little FABP content and no significant change with respect to development and is likely to have a relatively low capacity for β-oxidation of fatty acids. There is also little change in the FABP content of the heart muscle during development. This is in contrast to the results for the activity of HAD, in which there is a relatively large increase (ca. 10- to 15-fold) in enzyme activity in the heart muscle between hatch and fledging at 7 wk of age (5). Although the explanation of this result is not clear, it might suggest that FABP content is not a rate-limiting factor in influencing the oxidation of fatty acids in the developing heart. In the case of the pectoralis, however, it would appear that both the maximum rate of activity of the various mitochondrial enzymes and the FABP content are broadly co-regulated, such that FABP content increases directly with regard to the requirement to utilize fatty acid during muscle metabolism.

Long-term captive and wild postbreeding adult barnacle geese had lower FABP concentrations (~66%) in their pectoralis muscles than those of wild, pre-migratory geese. This would suggest that in physically mature geese, the experience of flight is important in maximizing FABP content in the flight muscles. The activity of CS was also lower (~75%) in these two groups of birds (5, 6). Thus, whereas developmental processes appear to be quantitatively the most important factor in the development of the aerobic capacity of various locomotor muscles, the experience of flight activity may be critically important in achieving maximal expression of FABP and associated mitochondrial enzymes in the pectoralis muscles of postfledging and mature geese immediately before migration.

Perspectives

The results of this study showed that there was a high degree of cross-reactivity (66%) of purified goose H-FABP with a set of Mabs directed against human H-FABP. This cross-reactivity indicates the presence of evolutionarily conserved epitopes, which is consistent with the fact that these two antibodies also react significantly with purified dog, bovine, rat, and mouse FABPs and with sparrow, crow, and starling homogenates of cardiac and pectoralis muscle. The evolutionary tree of H-FABP and the differences in tissue FABP content in the species studied can be linked to metabolic studies concerning fatty acid utilization versus physiological adaptation.

The fact that the FABP content in goose heart is less than expected cannot be easily explained. It would appear that FABP-mediated fatty acid transport is lower in barnacle goose heart than in the hearts of humans and rats. Indeed, it is speculated that avian cardiac muscle utilizes ketone bodies as an alternative source of lipid substrate, because this muscle is well adapted to ketone utilization in vertebrates and the maximum activities of the enzymes involved are much higher in the cardiac muscle compared with those in the pectoralis muscles of birds. It is not clear what percentage of the maximum oxygen consumption results in maximal lipid oxidation rates in the different tissues of these birds. If this percentage is relatively low in goose heart muscle, then this would also suggest that the metabolic pathways for fuel substrates are quite different in the heart compared with those of the pectoralis muscle.

The difference in FABP content in the pectoralis of captive and wild adult geese is very interesting. How can the experience of flight induce more premigratory FABP upregulation than that resulting from normal seasonal changes? Can it be that the stress of flight alters fuel mobilization or induces a stronger upregulation of fatty acid receptors, which induces an upregulation of FABP, and therefore that higher FABP content is found just before migration as the geese lay down a lot of fat? It is known that fatty acids and carbohydrates can influence gene expression. Further studies of bird metabolism and FABP tissue content may yield useful insights into fatty acid regulation and utilization during prolonged exercise.

The authors thank Prof. Dr. G. J. van der Vusse for stimulating discussions during this study and J. Willems for help with the illustrations.

Support for the field work was provided to P. J. Butler by Biotechnology and Biological Sciences Research Council Grant no. GR/F 82405.

Address for reprint requests: J. F. C. Glatz, Dept. of Physiology, Cardiovascular Research Institute Maastricht, Maastricht Univ., PO Box 616, NL-6200 MD Maastricht, The Netherlands.

Received 27 February 1998; accepted in final form 22 October 1998.

REFERENCES


