The Development of an Immunoblotting Assay for the Quantification of Liver Fatty Acid-Binding Protein During Embryonic and Early Posthatch Development of Turkeys (Meleagris gallopavo)

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ABSTRACT Turkey (Meleagris gallopavo) liver cytosolic fatty acid binding protein (FABP) was purified and used as a standard for quantification. An immunoblotting procedure was developed to study the ontogeny of liver cytosolic FABP during embryonic and early posthatch development in turkey poults. Liver FABP activity was also determined indirectly through the use of gel filtration chromatography followed by a ligand-binding assay. The specific activity of liver FABP (ng/mg of cytosolic protein) increased with length of incubation, peaking initially at Day 22, declining between Days 22 and 25, and increasing again from hatch (Day 28) to 6 d posthatch. The specific activity of liver FABP increased 12-fold between Day 13 of incubation and 6 d posthatch compared with total activity, which increased from 946 to 1.01 × 10⁶ ng/liver during the same period, a 1,067-fold increase. The results from both analytical procedures were similar, suggesting that the immunoblot method could be used to quantify liver FABP concentrations. The observed increases in FABP activity throughout the embryonic period and first days after hatching paralleled increases in liver lipid concentration. Therefore, liver FABP may be associated with hepatocyte fatty acid transport and metabolism during the latter stages of incubation and early posthatch period.

(Key words: turkey, poult, liver, fatty acid binding protein, lipid)

INTRODUCTION

Cytosolic fatty acid binding proteins (FABP) are small proteins with a high binding affinity for long-chain fatty acids (LCFA). These proteins are involved in intracellular fatty acid movement (Tipping and Ketterer, 1981; Ockner and Manning, 1982) and protection of enzymes from the toxic effects of free fatty acids (Glatz and Veerkamp, 1985). They may also play a role in the modulation of enzyme activity and signal transduction (Grinstead et al., 1983; Greer and Hargis, 1992; Glatz et al., 1993).

Several methods have been used to quantify the concentration and activity of tissue-specific FABP. Binding of radiolabeled LCFA by FABP after ammonium sulfate precipitation (Dutta-Roy et al., 1988) and protection of enzymes from the toxic effects of free fatty acids (Glatz and Veerkamp, 1985). They may also play a role in the modulation of enzyme activity and signal transduction (Grinstead et al., 1983; Greer and Hargis, 1992; Glatz et al., 1993).

Several methods have been used to quantify the concentration and activity of tissue-specific FABP. Binding of radiolabeled LCFA by FABP after ammonium sulfate precipitation (Dutta-Roy et al., 1988) and comigration of cytosolic FABP with radioactive LCFA on a gel filtration column (Ockner et al., 1972) are two procedures that have been used to measure FABP concentration. Cross-reactivity of labeled fatty acids with albumin and other proteins has been one problem associated with the ammonium sulfate precipitation procedure. Non-specific binding of radiolabeled LCFA to the matrix of gel filtration columns presents another potential problem associated with the comigration procedure. Subsequently, more specific methods have been developed that use the antigenic properties of purified FABP, such as immunochemical quantification (Ockner and Manning, 1982; Bass et al., 1985), radioimmunoassay (Fleischner et al., 1975), and ELISA (Paulessen et al., 1989; Ohkaru et al., 1994). The specificity of the antibody-antigen reaction allows for increased accuracy in the detection and quantification of FABP (Ockner and Manning, 1982; Epstein et al., 1994). One objective of the current study was to validate a procedure for the quantification of FABP without the complication of competitive binding from albumin. This procedure combined immunoblotting with computer scanning and color intensity analysis. The other objective was to compare the results of the immunoblot assay with a standard
ligand-binding assay. The results would confirm the validity of the ligand-binding assay for studying FABP in tissues for which a non-homologous immunooassay was unavailable.

Mammalian and chicken liver FABP have been well characterized. Rat liver FABP is approximately 14 kD with a pI of 8.1 (Ockner et al., 1974; Ockner and Manning, 1982; Glatz et al., 1985). Scapin et al. (1988) purified a basic form of chick liver FABP (16.5 kD; pI 9.0) whereas Sewel et al. (1989) described a different form of chick liver cytosolic FABP (14 kD, pI 7.0). In rats, hepatic FABP activity increases 20-fold from approximately 5 d before birth through 45 d of age (Sheridan et al., 1987). Gordon et al. (1985) reported that liver FABP mRNA levels were increased at 1 d of age but did not change during early life. In pigs, liver FABP activity is relatively constant from 73 d of pregnancy through birth (114 d) but then decreases through 7 d of age (Reinhart, 1990; Chi, 1993). There are no published data describing the ontogeny of liver FABP in avian species during late embryonic development and early posthatch growth. Turkey embryos use large quantities of fatty acids (Ding and Lilburn, 1996), and tissues that use fatty acids are sites for FABP expression (Glatz et al., 1993). An experiment was subsequently conducted to study the ontogeny of liver FABP in turkeys during embryonic development and the first few days after hatching. To this end, two methods were compared, immunoblotting and radioligand binding after gel filtration.

**MATERIALS AND METHODS**

Fertilized eggs from a commercial flock of Nicholas turkeys (*Meleagris gallopavo*) were incubated at the Ohio Agricultural Research and Development Center, Wooster, Ohio. Livers were collected from embryos and poults at 3-d intervals beginning at embryonic day (ED) 13 through 6 d posthatch (PD 6). Livers were excised from 8 to 16 individual embryos or hatched poults and pooled into one sample. Four replicate samples were collected from each stage of development. The liver samples were immediately washed in ice-cold saline (150 mM NaCl) containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) and were subsequently snap frozen in liquid nitrogen. The pooled samples were stored at -70 C until further analysis. Pooled samples were ground into a powder and homogenized in a 2x volume of 10 mM phosphate buffer (pH 7.4) containing 0.2 mM PMSF and 1 mM DTT. The homogenate was centrifuged at 10,000 × g for 15 min. The upper fat layer and pellet were discarded and the supernatant was further centrifuged at 59,000 × g for 2 h. The upper fat layer was again discarded and the supernatant was collected for FABP and protein analysis. The protein concentration was determined according to Bradford (1976) with bovine serum albumin as the standard. The concentrations of the subcellular enzyme markers pyrophosphatase, glucose-6-phosphatase, and succinate dehydrogenase were determined to ensure that the cytosol was not contaminated with debris from other subcellular fractions.

**Purification of Liver FABP**

Liver cytosol from 6-d-old poults was mixed with 70% ammonium sulfate at 4 C (Avanzati and Catala, 1983) and centrifuged at 4,000 × g for 5 min; the supernatant was desalted on a PD-10 column.4 The fraction thought to contain FABP was subsequently applied to a Sephadex G-75 column (1.75 × 75 cm), and 3-mL fractions were collected and analyzed for protein and FABP activity using a ligand-binding assay as described below. Fractions 24 to 32 had the greatest FABP activity and were subsequently pooled, concentrated to 4 mL, and dialyzed against Tris buffer overnight (30 mM, 9.0 pH). The solution was then applied to a DE 52 anion exchange column (1.75 × 25 cm) and washed with 40 mL of 30 mM Tris buffer (pH 7.4), followed by 60 mL of a 0 to 100 mM NaCl gradient containing Tris buffer. Finally, 40 mL of 500 mM NaCl containing Tris buffer was used to wash out all proteins. The flow rate was 0.5 mL/min, and 2.5-mL fractions were collected. Fractions containing FABP were subsequently combined. At each step, FABP in the samples was confirmed by Western blot analysis as described below.

**Confirmation of Liver Cytosolic FABP**

The immunoblotting procedure was a modification of the method of Burnette (1981). Hepatic cytosolic protein (25 µg) from each of the eight developmental stages, an internal standard (6.25 µg of cytosolic protein from 6-d-old poults), and a sample containing molecular weight markers were applied to a sodium dodecyl sulfate/polyacrylamide gel (12.5% wt/vol acrylamide). All samples were run in triplicate. Electrophoresis was performed for 2 h at 100 V using a dual vertical minigel apparatus.6 After electrophoresis, the gels were equilibrated in a transfer buffer (192 mM glycine; 25 mM Tris base; 20% vol/vol methanol) for 15 min. Proteins were transferred to 0.2-µm nitrocellulose membranes using a Hoefer electrophoretic transfer unit set at 150 mA; for 3 h after transfer, the membrane was dried and blocked for 30 min with 3% wt/vol BSA. The membrane was subsequently incubated for an additional 30 min with a 1:8,000 dilution of primary antibody specific for chick liver FABP (Collins and Hargis, 1989). The primary antibody cross-reacted with liver cytosolic FABP from turkey embryos and posthatch poults (Figure 1). After each immunoreaction, membranes were washed three times for 5 min with Tris-buffered saline-Tween solution (TBST; 20 mM Tris base with 0.05% vol/vol Tween-20; pH 7.50). Membranes were

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4 Wheaton, Inc., Millville, NJ.  
5 Pharmacia, Inc., Piscataway, NJ.  
6 Sigma Inc., St. Louis, MO.  
7 Harvard Inc., Cambridge, MA.  
8 Schleicher and Schuell, Keene, NH.  
9 Hoefer Inc., San Francisco, CA.
the internal standard to reduce variation between assays. The intensity value of each sample was adjusted according to applied to each gel to serve as an internal standard. The curve. Cytosolic protein (6.25 sample was calculated using the data from the standard subtracted from each reading. The FABP content of each band was reported in pixels, and the background readings were (9 to 147 ng/lane). The color intensity of each FABP band was measured twice, and background intensity for each band was deducted from the average of the two measurements. A standard curve was developed using a serial dilution of purified 6-d poult liver FABP for Western blot analysis. MW proteins were subsequently transferred to a nitrocellulose membrane sodium dodecyl sulfate, polyacrylimide (12.5%) gel electrophoresis, and proteins were subsequently transferred to a nitrocellulose membrane for Western blot analysis. MW = molecular weight marker. The hepatic cytosols from chickens, turkey pouls, and turkey embryos all had cross-reactivity with the antisera. The FABP activity was observed at the molecular weight of 14 kDa.

subsequently incubated for 30 min with a second antibody conjugated with alkaline phosphatase (1:10,000 rabbit antigoat IgG5). This step was followed by three subsequent 5-min washes in TBST and two brief rinses with a Tris-buffered saline solution (pH 7.5) to remove the Tween-20. Color was developed for 3 min in a mixture of 5-bromo-4-chloro-3-indoyl-phosphate and nitroblue tetrazolium.9 The reaction was stopped by rinsing the membrane with Tris buffer containing 10 mM EDTA (pH 7.0).

The procedure used for color intensity determination was a modification of the procedure described by Velleman (1995). The blot was scanned with a Hewlett Packard scanner using Deskscan II Version 2.0 software. The image was analyzed subsequently by SigmaScan10 to determine the color intensity of each band. The color intensity of each band was measured twice, and background intensity for each band was deducted from the average of the two measurements. A standard curve was developed using a serial dilution of purified 6-d poult liver FABP (9 to 147 ng/lane). The color intensity of each FABP band was reported in pixels, and the background readings were subtracted from each reading. The FABP content of each sample was calculated using the data from the standard curve. Cytosolic protein (6.25 µg) from PD 6 livers was applied to each gel to serve as an internal standard. The intensity value of each sample was adjusted according to the internal standard to reduce variation between assays.

**Functional Analysis of Liver FABP Activity**

The FABP activity of liver cytosol was also determined using the procedure of Morrow and Martin (1983) with the following modification. One milliliter of liver cytosol was prepared as described previously and applied to a Sephadex G-75 gel filtration column11 (1.75 × 75 cm; 1 mL/min; 3 mL/fraction) to separate FABP from other, larger proteins. Fractions containing FABP were collected and pooled.

The separation of large and small proteins had been validated in a previous assay by monitoring the separation of purified turkey serum albumin (TSA) from cytochrome C, a protein of the same approximate weight as FABP (data not shown). The pooled fractions used for FABP determination were free of TSA. The existence of liver FABP in these fractions was positively identified by Western immunoblot analysis as previously described. Fractions corresponding to the molecular weight of FABP were pooled for each sample. FABP activity in embryonic liver was low so all samples were concentrated 2.5x by lyophilization. FABP activity was subsequently determined via the ligand-binding procedure of Glatz et al. (1984) using Lipidex 5000. FABP activity was estimated as disintegrations per minute (dpm)/mg of cytosolic protein or dpm/organ.

**Statistical Analyses**

Linearity of the calibration curves was tested using the PROC REG procedure of SAS software (SAS Institute, 1985). A t-test was used to test for parallelism between the two standard curves generated from the serial dilutions. The ontogeny data was analyzed by one-way analysis of variance using the general linear models procedure of SAS software (SAS Institute, 1985). Age was the main effect tested, and all values are reported as means ± standard error of the mean.

**RESULTS**

The body weight of embryos increased from 4.16 g at ED 13 to 52.3 g at hatch (ED 28) and from 71.6 g at ED 3 to 104.1 g by PD 6 (Table 1). The liver weighed 67 mg at ED 13, 1.57 g at hatch (ED 28), and 4.12 g at 6 d of age.

**TABLE 1. Changes in body and liver weights during embryonic development and early postnatal growth of turkeys (Meleagris gallopavo)**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Embryo number</th>
<th>Body weight g</th>
<th>Liver weight g</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED 13</td>
<td>32</td>
<td>4.16 ± 0.02</td>
<td>0.067 ± 0.002</td>
</tr>
<tr>
<td>ED 16</td>
<td>64</td>
<td>9.59 ± 0.17</td>
<td>0.18 ± 0.003</td>
</tr>
<tr>
<td>ED 19</td>
<td>64</td>
<td>19.44 ± 0.34</td>
<td>0.34 ± 0.003</td>
</tr>
<tr>
<td>ED 22</td>
<td>32</td>
<td>29.44 ± 1.27</td>
<td>0.59 ± 0.010</td>
</tr>
<tr>
<td>ED 25</td>
<td>32</td>
<td>42.94 ± 0.66</td>
<td>0.78 ± 0.020</td>
</tr>
<tr>
<td>ED 28</td>
<td>32</td>
<td>52.31 ± 1.14</td>
<td>1.57 ± 0.050</td>
</tr>
<tr>
<td>PD 3</td>
<td>24</td>
<td>71.56 ± 1.75</td>
<td>2.69 ± 0.100</td>
</tr>
<tr>
<td>PD 6</td>
<td>24</td>
<td>104.12 ± 2.18</td>
<td>4.12 ± 0.110</td>
</tr>
</tbody>
</table>

Probability of age effect 0.0001 0.0001

1ED 13 to 28 = embryonic day 13 to 28. ED 28 is the hatching day. PD 3 to 6 = 3 to 6 days posthatch.
2Mean ± SEM.

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Promega, Madison, WI.

Jandel Scientific, San Rafael, CA.

**FIGURE 1.** Cross-reactivity of goat anti-chick liver fatty acid binding protein (FABP) antisera with hepatic cytosol samples from broiler chicks (4 wk of age), turkey pouls (6 d of age), and turkey embryos (22 d of incubation). Hepatic cytosol aliquots (20 µg protein) were subjected to sodium dodecyl sulfate, polyacrylimide (12.5%) gel electrophoresis, and proteins were subsequently transferred to a nitrocellulose membrane for Western blot analysis. MW = molecular weight marker.
Liver cytosol was treated with 70% ammonium sulfate solution and desalted by PD 10 column before it was applied on a Sephadex G-75 column (1.75 × 75 cm). Fractions eluted with 10 mM phosphate buffer (pH 7.4) were collected at 1 mL/min and 3 mL/fraction. A) Protein concentration and fatty acid binding protein (FABP) activity (act.) of collected fractions. OD280 = optical density at 280 nm. B) Proteins were detected on sodium dodecyl sulfate, poly-acrylimide (12.5 %) gels by silver staining. MW = molecular size markers; 24-32 = pooled samples from fractions 25 to 32. C) Western blot analysis of partially purified liver FABP. Cross-reactivity of the protein with chick liver FABP antibody was observed.

Purified liver FABP from turkey poult had approximately the same molecular weight (14 kD) as chicken liver FABP (14.5 kD) and exhibited strong cross-reactivity with chicken liver FABP antiserum (Figure 1). Liver FABP was purified from PD 6 turkey poult, and the protein concentration, FABP activity, and FABP activity in the fractions collected off the gel filtration column are shown in Figure 2. There were two peaks with respect to fatty acid binding activity (Figure 2a). The existence of small molecular weight proteins associated with fractions 24 to 32 was confirmed by silver staining (Figure 2b), and FABP specificity was confirmed by Western immunoblot (Figure 2c). Fractions 24 to 32 were pooled for subsequent ion exchange chromatography. A sample of the first pre-gradient peak (fractions 12 and 13; Figure 3a) eluted from the ion exchange column had a single protein at approximately 14 kD (Figure 3 b), and the existence of FABP was confirmed after a Western immunoblot with anti-chick liver FABP antiserum (Figure 3c). These fractions were subsequently pooled and used in development of a standard curve for FABP determination. The standard curve was linear (r = 0.983; P ≤ 0.05; Figure 4). The ontogeny of liver FABP, as visualized in a typical Western immunoblot and expressed in nanograms as calculated from the FABP standard curve, is shown in Figure 5a. The ontogeny of liver FABP, as determined by immunoblotting and expressed in nanograms as calculated from the FABP standard curve, is shown in Figure 5b. An age effect (P ≤ 0.0001) was observed for FABP concentration when calculated either as nanograms per milligram of cytosolic protein (specific activity) or nanograms per total liver (total activity). A trace amount of FABP was detected on ED 13, and specific activity increased with length of incubation. Overall, a 12-fold increase in liver FABP specific activity was observed between ED 13 and PD 6. Total activity increased continually from 946 ng on ED 13 to 1.01 × 10⁶ at ED 6, a 1,067-fold change. The ontogeny of liver functional FABP activity determined via binding of ¹⁴C oleic acid after gel filtration was similar in slope to the Western blot results (Figure 6). Specific activity determined with the ligand-binding assay (dpm/mg cytosolic protein) increased between ED 16 and PD 6, a 10-fold increase. Total activity (dpm/liver) increased 286-fold during the same period.

The immunoblot procedure described herein was useful for visualizing changes in liver FABP concentration.

FIGURE 2. Liver cytosol was treated with 70% ammonium sulfate solution and desalted by PD 10 column before it was applied on a Sephadex G-75 column (1.75 × 75 cm). Fractions eluted with 10 mM phosphate buffer (pH 7.4) were collected at 1 mL/min and 3 mL/fraction. A) Protein concentration and fatty acid binding protein (FABP) activity (act.) of collected fractions. OD280 = optical density at 280 nm. B) Proteins were detected on sodium dodecyl sulfate, poly-acrylimide (12.5 %) gels by silver staining. MW = molecular size markers; 24-32 = pooled samples from fractions 25 to 32. C) Western blot analysis of partially purified liver FABP. Cross-reactivity of the protein with chick liver FABP antibody was observed.
during development and for quantifying the developmental differences. Moreover, the immunoblot procedure avoided problems associated with sample contamination with albumin or other proteins that have a binding affinity for LCFA. The specificity of the antigen-antibody reaction enhanced the accuracy of the assay, and variability was reduced when samples from each developmental stage could be included in the same blot. Similar results obtained from the immunoblot and ligand-binding assays further support the validity of the immunoblot procedure, but they also lend support to the use of the ligand-binding assay should antisera to tissue-specific FABP not be available.

In the present study, the concentration of liver FABP increased throughout embryonic development and after hatching. These increases correlate well with documented increases in liver lipid and the active transfer of fatty acids from the yolk sac to the embryo during the course of incubation (Ding et al., 1995; Ding and Lilburn, 1996). This finding suggests that in turkey embryos, FABP may play a role in cytosolic fatty acid translocation. This pattern of embryonic development is somewhat different than what has been reported in other species. In swine, liver FABP is high in the fetal liver but then decreases during the first week after birth (Chi, 1993; Reinhart, 1990). In rats, Sheridan et al. (1987) reported that liver FABP was 20-fold higher in fetal liver compared with adults. Species differences are most likely a reflection of differences in fatty acid metabolism during embryonic and early postnatal growth. In the chick liver, the activity of enzymes involved in lipid metabolism also increase during the latter stages of incubation (Linares et al., 1993; Shand et al., 1994), and it has been suggested that liver FABP stimulates acyl CoA:cholesterol acyltransferase (ACAT) activity (Greer and Hargis, 1992). The enzyme ACAT is responsible for the esterification of fatty acids to cholesterol, primarily within the yolk sac membrane but also in the embryonic liver of chicks (Shand et al., 1994) and pouls (Ding and Lilburn, 2000). Ding and Lilburn (1996) reported that lipid subclass concentrations and fatty acid profiles in turkeys are similar to what has been reported for chickens so it could be surmised that FABP is playing a similar role in turkey embryos. The increase in liver FABP from hatch through PD 6 is most likely a reflection of the major changes in liver fatty acid flux during this period. At hatch, hepatic cholesterol ester concentrations are extremely high in turkey pouls (Ding and Lilburn, 1996), and fatty acids derived from cholesterol ester hydrolysis serve as a major metabolic fuel (Noble and Cocchi, 1990). To this end, liver FABP may serve as a temporary sink for newly hydrolyzed fatty acids as
FIGURE 5. A) The ontogeny of liver cytosolic fatty acid binding protein (FABP) as visualized in a typical Western blot. ED 13 to 25 = embryonic days 13 to 25; H = hatch; PD 3 to 6 = posthatch days 3 to 6. Each lane contained 25 µg of total liver cytosolic proteins. B) Ontogeny of liver FABP during embryonic and early postnatal growth in turkeys by using Western blot analysis. Error bars indicate SEM. Color intensity of each band was scanned and analyzed with HP Deskscan II Version 2.0 software and SigmaScan (Jandel Scientific, San Rafael, CA), respectively. The standard curve of purified FABP was used. Conc. = concentration.

FIGURE 6. Ontogeny of liver fatty acid binding protein (FABP) activity during incubation and early postnatal growth in turkeys. Error bars indicate SEM. Liver cytosols were subjected to a Sephadex G-75 gel filtration column (1.75 × 75 cm) eluted with 0.01 M phosphate buffer (pH 7.4; 4 C, 60 mL/h). Fractions corresponding to an elutant volume of 72 to 99 mL were pooled for FABP activity determination. 14C-oleic acid was the ligand used for FABP activity determination. DPM = disintegrations per minute; ED 16 to 19 = embryonic days 16 to 19; H = hatch; PD 3 to 6 = posthatch days 3 to 6.
well as a transport vehicle for fatty acids to the site of \(\beta\)-oxidation.

In conclusion, a novel procedure was developed for quantifying concentrations of liver FABP. The procedure combined existing immunoblot methodology with computer scanning and color intensity analysis. The results from this analysis were similar to those from a standard ligand-binding assay, and both assays showed that increases in liver FABP were consistent with observed increases in liver lipid concentration and fatty acid metabolism in the turkey poult.

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