THE ROLE OF CHICK EMBRYO HEPATIC FATTY ACID BINDING PROTEIN IN LIPID METABOLISM

A Senior Thesis

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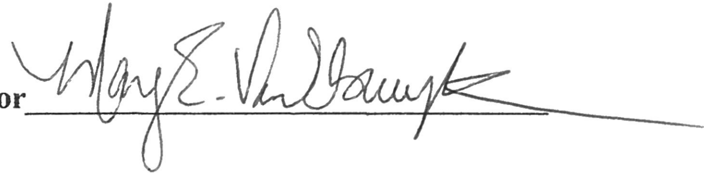
Fellows Advisor 

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Abstract

Liver fatty acid binding protein (L-FABP), an intracellular binder of free fatty acids, is believed to play a role in the targeting of fatty acids towards synthetic and/or oxidative pathways. The chick, with its dramatic shifts in energy demands during growth/development and hatch may serve as an excellent model to more clearly delineate L-FABP function. The purpose of the current study was to investigate the expression of chick L-FABP throughout embryogenesis and early development. L-FABP expression was measured at days 10, 14, and 17 of embryogenesis and days 1, 4, 7, and 11 post-hatch using western blot analysis with goat anti-chicken L-FABP as the primary antibody. The L-FABP protein bands were visualized using chemiluminescence and quantified using densitometry. L-FABP expression increases dramatically throughout embryogenesis and early post hatch. The greatest increases in L-FABP expression occurred during the key transition between early embryogenesis and hatch, increasing an average of 30 times from d10 to d21 (day of hatch). It may be suggested from the results of the current study, therefore, that L-FABP plays a major role in increasing the availability of liver fatty acids for oxidation during the critical transition period of the embryo as a poikilotherm to a homeotherm. Further studies, designed to correlate L-FABP expression with specific enzymes involved in the oxidation of fatty acids are needed to confirm this theory.

Literature Review

Diets high in saturated fat and cholesterol are believed to increase the risk of coronary heart disease, but this does not mean that all fats are bad. Epidemiological studies have shown that the consumption of polyunsaturated fatty acids (PUFA) may reduce the risk of heart disease (Leaf and Weber, 1988). Omega 3 (n-3) fatty acids and omega 6 (n-6) fatty acids are the two classes of PUFA that exist. The omega nomenclature system helps determine the characteristic properties and structure of PUFA. For example 22:5 n-3 is eicosapentaenoic acid. The "22" refers to the number of carbons in the fatty acid (FA) chain. The "5" refers to the number of double bonds which determines the degree of unsaturation. An increasing number of double bonds increases the degree of unsaturation and no double bonds means the fatty acid is saturated. The "n-3" indicates the position of the first double bond. The carbon chain of a fatty acid has a methyl end with the first carbon referred to as the omega carbon. The first double bond in this example is on carbon number three from the methyl end and thus it is termed an omega-3 or n-3 fatty acid. The omega-6 FA linoleic acid (LA; 18:2 n-6) and the omega-3 FA linolenic acid (LNA; 18:3 n-3) are considered nutritionally essential because humans are unable to synthesize fatty acids with double bonds distal to the ninth carbon from the carboxyl end of the FA. Grain fed beef products, typically high in the US diet, serve as sources of n-6 FA. On the other hand, marine food sources and flaxseed serve as sources of n-3 FA. Unfortunately, these food are limited in the US diet (USDA, 1994).

Epidemiological studies indicating promotion of heart health from n-3 FA

consumption suggest that two 3 oz. (6 oz. total) fish meals a week are sufficient to result in expected benefit (Bang and Dyerberg, 1972). Despite the promotion of fish consumption, USDA per capita fish consumption data indicates that Americans are still consuming less than 6 oz. of fish per week. In 1970, US fish consumption averaged 3.6 oz. per week. By 1993 consumption had risen to 4.6 oz. per week which still falls below the recommended amount (USDA, 1994). Reasons for these finding might be the lack of availability of fish or the high cost of fish. Another reason might be many people don't like the taste of fish. Whatever the reason, health professionals have recommended the production of food-based alternatives of n-3 FA to increase n-3 FA availability.

Dietary FA modification may be a viable means of producing n-3 FA rich foods. In poultry, for example, the fat composition of the hen's diet is readily reflected in the yolk (Cruickshank, 1934). The shell egg therefore is a candidate for n-3 FA enrichment. Additionally, shell eggs are an economical food as well as a nutrient dense food. Eggs are also widely available and readily consumed so that a large portion of the population would have the opportunity to consume these desirable FA. Feeding laying hens a diet rich in n-3 FA results in table eggs with a similar amount of n-3 FA in the yolk as a lean serving of fish. For example, Hargis et al., (1991) fed hens 3.0% menhaden oil for 3 months and reported approximately 220 mg/egg of total n-3 FA. A typical serving of lean fish contains about the same. Therefore, by replacing regular eggs with n-3 FA enriched eggs, consumers have a better chance of meeting n-3 FA intake goals as Americans still consume slightly over three eggs per week (TAES, 1993).

While these changes in laying hen feeding regimes to include n-3 FA may benefit the consumer, the health of the hen must also be considered. Including n-3 FA in laying hen diets may adversely impact hen health because mammalian studies suggest that the beneficial hypolipidemic effect of the n-3 FA is a result of decreased synthesis and secretion of liver lipid. The hen, under the influence of estrogen, produces about 5 grams of triglyceride in the liver daily for yolk deposition. Given this high rate of lipid synthesis, a dietary modification that impacts secretion could have a profound influence on liver lipid homeostatic mechanisms. Coincidentally, one problem with the n-3 FA enriched laying hen diet is the higher incidence of accumulation of lipids in the laying hen liver. This accumulation of fat in the liver can predispose laying hens to the development of a metabolic disease of laying hens known as Fatty Liver Syndrome (FLS). FLS was first described by Couch in 1956 as he searched for an explanation for a decrease in egg production among his laying hen flocks. Although he found no overt disease process, grossly, he noted a pale, enlarged, friable, putty colored liver, owing to the amount of fat deposited in the liver. FLS can precede a more pathological condition know as Fatty Liver Hemorrhagic Syndrome characterized by increased hepatic triglyceride content associated with liver hemorrhages and hematomas that usually result in a significant drop in egg production (Wolford and Polin, 1972; Hansen and Walzem, 1993).

Proposed etiologies for FLS include excess dietary energy, estradiol, and dietary menhaden oil (fish oil). The high CHO diet of laying hens is known to be very lipogenic, and this lipogenesis coupled with the decreased activity of the laying hen due to a caged

environment can result in a net accumulation of liver lipid. Lipogenesis also increases substantially in response to increased production of estrogen as the hen prepares to lay eggs. This increase in production of estrogen at the onset of egg laying is coupled with an increase in liver fatty acid binding protein (L-FABP) mRNA. Treatment of laying hens with exogenous estrogen also increases the degree of hepatic lipid infiltration. Van Elswyk and co-workers (1994) compared feeding diets containing 3% menhaden oil (MO) or 3% animal/vegetable (AV) oil to both sexually immature and reproductively active male and female chickens. They concluded that dietary menhaden oil and estradiol appear to interact in a manner that enhances the lipogenic activity of the liver, thereby inducing hepatic lipidosis in laying hens. They noted no differences in hepatic lipid accumulation between males fed 3% MO diets or those containing 3% AV oil. All of the livers in the males were normal histologically; however, hens fed the 3% MO had a significant amount of hepatic lipid infiltration compared to the hens fed 3% AV. This observation of hepatic lipidosis led to the convergence of two lines of research in our laboratory. Specifically, we had previously isolated two lipid transport proteins from chicken liver: 1) non specific lipid transport protein (ns-LTP) also referred to as sterol carrier protein-2 (SCP-2) and 2)L-FABP (Sams et al, 1991). Studies in the lab have found that fatty livers have a high amount of fatty acid binding protein (McNeill, unpublished data).

To move throughout the body, FA must be carried by proteins. Albumin is the protein responsible for carrying fatty acids in plasma. Intracellulary, FABP are

responsible for the transport of FA. FABP are a family of low molecular weight (14-15 KDa) cytosolic lipid binding proteins that are found in a variety of tissues known to actively metabolize lipids including the small intestine, liver, adipose, heart, brain, muscle, kidney and arteriole wall (Matarese et al, 1989). FABP are highly conserved among species and are believed to have been present prior to the emergence of vertebrates (Chan et al. 1985). Liver fatty acid binding protein (L-FABP) is the most abundant of these proteins and is expressed in both the liver and small intestinal epithelium. L-FABP have been shown to form 2-5% of the cytosolic proteins in these tissues (Bass and Manning, 1986; Kaikaus et al, 1993). L-FABP has a high affinity for long chain FA. Affinity appears to increase as chain length and degree of unsaturation increases. Up to 85% of FA endogenously bound to rat L-FABP are unsaturated with a high percentage (50-75%) of polyunsaturated species (Cistola et al, 1989; Ockner et al, 1982).

L-FABP relationship to FA is unclear. The binding of free FA to L-FABP may function to protect the cell from the potential deleterious effects of high concentrations of free long chain fatty acids on membrane integrity (Brenner, 1984). The high concentration of cytosolic L-FABP may also indicate that they may be important in the temporary intracellular storage of FA. It is known that L-FABP are responsible for facilitating the intracellular transport of FA, however their role in FA metabolism is vague. Existing literature is controversial concerning L-FABP's role in directing FA toward synthetic or oxidative (energy producing) pathways. Evidence of FABP's role in the synthesis of complex lipids for synthesis is suggested by Wu-Rideout and co-workers

(1972). The authors used isolated hepatocytes to investigate FABP's role in the direction of long chain FA toward microsomal esterification and subsequent triglyceride formation. They noted that flavaspidic acid, a competitor with free FA for binding to FABP, inhibited microsomal fatty acid activation and stimulated mitochondrial fatty acid activation which is the reverse of the effects of FABP. On the other hand, there is experimental data which indicates that rat L-FABP may direct metabolism of FA toward beta oxidation for energy production. For example, Applekvist and Dallner (1980) noted the presence of L-FABP in liver peroxisomes a site of oxidation of long chain FA. Unfortunately, the scientific data supporting a definitive role of L-FABP in either fatty acid oxidation or esterification still remains unclear.

Enhanced lipid infiltration in laying hen liver is influenced by a number of factors including two that also affect L-FABP expression: hormones and diet. In the chicken, the increased production of estrogen at the onset of egg laying is coupled with an increase in L-FABP mRNA. Treatment of laying hens with exogenous estrogen also increases the degree of hepatic lipid infiltration. Because of this effect of estrogen, L-FABP is believed to be sexually dimorphic. Actually, FABP has been shown to be sexually dimorphic in the rat with females expressing 1.5-2 times the amount of L-FABP as males (Bass and Manning, 1986). An increase in dietary fat also increases L-FABP. It has been proposed that this hepatic accumulation of fat is related to menhaden oil's affect on L-FABP expression. The chick, with its dramatic shifts in energy demands during growth/development and hatch, may serve as an excellent model to more clearly delineate

L-FABP function. Understanding L-FABP function should help interpret data currently being reviewed by our lab indicating increased FABP expression with fatty liver (Herber-McNeill and Van Elswyk, 1996).

The chicken provides an excellent model in which to study lipid metabolism and transport. Chicken embryos obtain an estimated 90% of their total energy requirements from the oxidation of yolk sac lipids (Noble, 1987). The nutrients of the yolk sac are not subject to change and can be quantified without difficulty. Additionally, the chick embryos are not subject to uterine/maternal influences. Finally, de novo lipogenesis in both the laying hen and human is confined to the liver. This is significant because, in the laying hen most of the lipid synthesized is directed toward yolk production of the egg. This is in contrast to the rat which is a model in many L-FABP studies where de novo fatty acid synthesis occurs in both liver and adipose tissue.

Materials and Methods

This research was conducted in a series of three trials. Each trial utilized the same methodology as described below.

Incubation

Dekalb strain Single Comb White Leghorn fertilized eggs were ordered from Feathercrest Farms (Bremen, AL, 35033). For each trial, eggs were incubated in a Petersime model S-11 setter with the large end of the egg pointing up so the head of the chick would develop at the large end of the egg near the air cell. A dry bulb temperature of 99.5 °F and a wet bulb temperature of 87 °F were maintained so relative humidity was approximately 58-60% which is essential for the proper development of the chick embryo. Eggs were rotated at 40-45 degree angles and one turn was completed every 1.5 hours by a mechanical turner. The turner in the incubator prevented the embryos from sticking to the side of the shell. At day 18 of incubation, the eggs were moved to a Petersime model S-1 hatcher. After hatch the chicks were moved to a Petersime six level chick brooder unit. The chicks were placed on chick starter feed the third day after hatch. Prior to diet initiation, the chick uses nutrients from the egg yolk that are encapsulated within the abdomen just before hatch.

Tissue Sampling

Livers were excised on days 8 (second and third trials only), 10, 12, 14 and 17 of incubation. Samples were also taken on days 1, 3, 7 and 11 post-hatch. All chicks and embryos were humanely handled and properly euthanized before sampling. Due to requirements of the FABP assay, at least one gram of liver was needed for each sampling day. For samples taken on

day 8 of incubation the embryo was placed in a dish of .1M phosphate buffered saline (PBS) with a pH of 7.4 at 4 °C. Livers were then floated out by "teasing" the liver away from the body. Trial one did not have a sampling on day 8 because this floatation method was not learned until after trial one was completed. Based on average liver weight, approximately 200 livers (– 15 mg each) were collected at day 8, approximately 40 livers (– 29 mg each) were collected at day 10. The number of livers collected for each sampling day continued to decline as the livers increased in weight; however, to maintain adequate representation within days each pool contained a minimum of 10 livers.

Cytosolic Protein Determination

For each sampling, livers were collected in conical tubes with PBS and stored at -75 °C until further analysis. Prior to analysis, each pool was thawed and homogenized for 3 minutes in 2.5 volumes PBS with a Precise® Virtis (Precise Products Corp, Racine, Wisconsin, 53401) at speed 30 on the virtis dial. The pools were then centrifuged at 800 g for 15 minutes with a Sorvall RT6000B table top centrifuge. The supernatant was collected and centrifuged at 105,000g for one hour and fifteen minutes with a Sorvall T1270 rotor (DuPont Medical Products, Newtown, CT, 06470). The pure cytosol (supernatant) was placed in eppendorf tubes and stored at 4 °C. A bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford Il., 06470-5509) was used to determine total cytosolic protein concentrations of the samples. Cytosolic proteins were diluted with Sample Application Buffer (SAB) to a final concentration of 2.5 or 5 µg/µl.

SDS Page and Western Blot Analysis

Twenty five µg of total cytosolic protein for each sample was separated by Sodium

Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) using gels containing 17% polyacrylamide in the separating layer. The electrophoresed protein was then transferred to a polyvinylidene fluoride (PVDF) membrane. Each PVDF membrane was blocked overnight in approximately 400 mls of 5% Carnation Nonfat Dried Milk in tris buffered saline with tween 20 (TBST) at 4 °C. After blocking of the PVDF membrane non-specific binding sites, the membranes were incubated in a solution containing the primary antibody consisting of goat anti-chicken L-FABP in TBST at a concentration of 1:500. The membranes were subsequently washed 3 times for 5 minutes each in TBST. After the last wash, 90 mls of secondary antibody, consisting of a 1:90,000 concentration of rabbit anti-goat IgG in TBST was added to the membrane and incubated for 1.5 hours. Secondary antibody was washed from the membrane using 3, 5 minute washes in TBST followed by 2, 5 minute washes in TBS.

L-FABP protein bands were visualized by incubating the membranes in 30 mls of the Pierce Super Signal Substrate Western Blotting solutions (kit #34080) with the membranes for 5 minutes. Next, the membranes were exposed to Kodak Biomax MR scientific imaging film (Eastman Kodak, Rochester, NY 14650) for 35 seconds in an Amersham Life Science Hypercassette™ (Amersham Life Science Inc., Arlington Heights, IL, 60005). A Konica QX-70 Medical Film Processor was used to develop the film. The amount of L-FABP was quantified using densitometry and the results were given in the unit OD x mm^2.

Results and Discussion

Liver Weights

As would be anticipated liver weights increased significantly ($P < .001$) during the transition between incubation and early post-hatch life. During incubation significant changes in liver weight were only observed between the early and late stages of embryonic development. Specifically, liver weights were not significantly different in the early embryonic period (d10-d14) but increased significantly during the late embryonic period immediately prior to hatch (d14 -d17) (Table 1). Between d17 and d21, which marks the transfer from incubation to hatch, was represented by another significant increase in liver weight (Table 1). At each point investigated during the neonatal period the pattern of liver weight increase continued to be significant as would be expected during this period of rapid growth and development.

TABLE 1. Liver Weights During Embryogenesis and Early Post-Hatch

Day	
10	$.027 \pm .001^{f}$
12	$.069 \pm .003^{f}$
14	$.138 \pm .006^{f}$
17	$.357 \pm .011^{e}$
21	$.677 \pm .045^{d}$
24	$1.04 \pm .033^{c}$
28	$2.25 \pm .090^{b}$
32	$3.82 \pm .086^{a}$

[ab]Means ± SEM differ significantly if superscripts differ

L-FABP Expression

The limited supply of hepatic material for each day of incubation/post-hatch life allowed for only one liver pool to represent each sampling in the evaluation of L-FABP expression during each trial. This situation prohibited the statistical evaluation of an interaction between trials and days therefore the trials are presented independently (Tables 2-4).

TABLE 2. L-FABP Expression (OD x mm^2) During Embryogenesis and Early Post-Hatch Life - Trial 1

Day	
10	.1106
12	-
14	.4853
17	.6454
21	.6744
24	1.239
28	1.970
32	2.891

TABLE 3. L-FABP Expression (OD x mm^2) During Embryogenesis and Early Post-Hatch - Trial 2

Day	
8	.1806
10	.0539
12	-
14	.5322
17	.5530
21	1.709
24	2.238
28	2.931
32	2.838

TABLE 4. L-FABP Expression (OD x mm^2) During Embryogenesis and Early Post-Hatch - Trial 3

Day	
8	-
10	.0434
12	.1994
14	.8279
17	.6240
21	.9839
24	1.537
28	2.181
32	-

Changes in L-FABP expression were modest during the period of embryogenesis with the exception of the changes between d10 and d14, or early embryogenesis (Tables 2-4). Likewise changes in L-FABP expression during early post-hatch life were also modest. The greatest increases in L-FABP expression, therefore, occurred during the key transition between early embryogenesis and hatch, increasing an average of 30-times from d10 to d21 (day of hatch). Avian embryo energy metabolism experiences a tremendous shift immediately prior to hatch. During the majority of incubation the embryo is poikilothermic and therefore unable to control energy metabolism for heat production (French, 1997). Studies have shown, however, that about d18 of incubation the chick embryo can maintain oxygen consumption in response to shifts in incubation temperature (Tazawa et al., 1989). After pipping, or initiation of hatch, a shift toward independent thermoregulation is observed and this ability to efficiency metabolize energy to maintain heat production in response to cold stress continues to improve after hatch (French, 1997). If indeed FABP is primarily involved in oxidative activities it would be anticipated that as the embryo becomes more able to regulate energy metabolism the need for FABP expression would increase. It may be suggested from the results of the current study, therefore, that L-FABP plays a major role in increasing the availability of liver fatty acids for oxidation during the critical transition period of the embryo as a poikilotherm to a homeotherm. Further studies, designed to correlate L-FABP expression with specific enzymes involved in the oxidation of fatty acids are needed to confirm this theory.

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