Effect of Feed Restriction and Dietary Fat Type on Liver Fatty Acid Binding Protein mRNA Expression in the Broiler Chickens

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***Abstract*—Liver fatty acid binding protein (L-FABP) is the main cytosolic binding site for long chain fatty acids in hepatocytes. FABPs enhance uptake of fatty acids into the cell by increasing their concentration gradient, due to minimizing unbound fatty acid in the cell. A total of 720, 10-day old male Ross 308 broiler chicks were fed diets with unsaturated to saturated fatty acid ratio (U/S) of 2, 3.5, 5 or 6.5 as ad lib or skip a day feeding schedule (during 18-28 days of age). The results clearly showed that feed restriction induced L-FABP gene expression in the livers of broiler chickens. The L-FABP gene expression increased by dietary unsaturated to saturated fatty acid ratio of 6.5. No interaction of dietary U/S and feed restriction on the liver L-FABP gene expression was observed. This observation proposes that birds have a mechanism for regulation of fatty acids transfer under different nutritional condition.**

***Index Terms*—L-FABP, real time PCR, feed restriction, dietary U/S, broiler chickens**

I. Introduction

Because of limited capacity of digestive tract, plant or animal fats or their mixtures are important components of broilers high-energy diets. Fats with high-unsaturated fatty acid content have a more absorbability and there is a known synergism between saturated and unsaturated fats [1], [2]. Age of birds affects fat digestibility too, because at earlier ages, there is an inadequate production of fat digestive enzymes from liver [3]. Fat metabolism is under exact control and several genes are involved.

The intracellular fatty acid-binding proteins (FABPs) comprise a family of 14-15 kDa proteins which bind long-chain fatty acids [4], [5]. Members of this family have been evolved over approximately one billion years by subsequent duplication and diversification of an ancestral intracellular lipid binding protein gene, thereby generating a large number of tissue-specific homologs [6].

The FABPs may modulate lipid metabolism via an involvement in the fatty acid uptake or export process, by regulation of substrate and/or product concentrations in the cytosolic compartment as a whole or more locally near particular enzymes, and/or by specifically delivering or removing fatty acids to/from particular enzymes [7]. FABPs enhance uptake of fatty acids into the cell by increasing their concentration gradient, due to minimizing unbound fatty acid in the cell [8].

It is suggested that L-FABP may function in the partitioning of fatty acids to different lipid metabolic pathways [6]. The control of tissue-specific expression of the various FABP types is only poorly understood. Often, the expression of FABPs in a given tissue reflects its lipid metabolizing capacity and increased fatty acid exposure leads to a marked increase in FABP expression [9].

Liver have a important role in fatty acid absorption [10], [11], directing fatty acids to particular metabolic pathways [6], lipoprotein synthesis [12] and transport of peroxisome proliferator-activated receptor ligands to the nucleus and consequential modulation of target-gene expressions [13].

The basic liver type fatty acid binding protein (Lb-FABP) is the only FABP that is not expressed in mammals. It is found in the liver of birds, fish, reptiles, and amphibians [14]. In chickens, the Lb-FABP gene is expressed only in the hepatocytes, whereas the L-FABP expression is done in both liver and intestinal tissues [15]. In the Ref [15], only little amounts of the L-FABP and Lb-FABP mRNAs were identified in the liver during chicken embryogenesis, but at the onset of hatching a remarkable increase in mRNA expression was detected for both genes, suggesting that the expression of the L-FABP and Lb-FABP genes is coordinated at developmental stages [15].

The L-FABP expression alters by a number of factors that greatly impact hepatic fatty acid metabolism, including feed restriction, high-fat diets and peroxisome proliferators [16], [17]. Ref [18] showed a dose-dependent increase in L-FABP gene expression of the chickens fed soybean lecithin.

Ref [19] showed that feed restriction reduced the expression of genes involved in lipogenesis, but enhanced

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the expression of genes like L-FABP which involve in fatty acids transport, in hepatocytes of broiler breeder chickens. Ref [15] showed that feeding motivation was an important factor inducing Lb-FABP gene expression. In contrast, feeding stimulation only somewhat encouraged expression of the L-FABP gene, and was not at all times its main determinant.

The present study aimed to describe the evolution effects of feed restriction and dietary fat saturation degree on liver fatty acid binding protein gene expression in male broiler chickens.

II. Materials and Methods

Seven hundred and twenty, 10-day old male chicks (Ross 308) were used in the study. The birds were randomly assigned according to their initial body weights to a completely randomized design with a 2×4 factorial arrangement with 3 replicates. Experiment factors were: 1- skip a day or free feeding at days 18-28 of age and 2- diets with different unsaturated to saturated fatty acid ratios (2, 3.5, 5 and 6.5) formulated using different levels of sunflower oil and beef tallow. Fatty acids content of beef tallow and sunflower oil determined by gas chromatography. The birds were fed a grower diet until 28 days of age followed by a finishing diet at 29 to 42 days of age. All birds were handled in accordance with the Ross 308 broiler chickens Management Guide specifications. The ingredients and the composition of experimental diets are shown in Table I.

At the end of grower (28d) and finisher (42d) periods, four broilers per treatment selected at random, were slaughtered by decapitation and the liver sections were rapidly dissected out and flash-frozen in liquid nitrogen and stored at −80◦C. Total RNA of each tissue sample was extracted by TRIZOL Reagent kit (Invitrogen Inc., CA) according to manufacturer's instruction. The integrity and purity of RNA were tested by measurement of optical density (ratios at 260 and 280 nm being greater than 1.9) and by electrophoresis using ethidium bromide staining. Total RNA was treated by RNase-free DNase I (Roche, Mannheim, Germany) and was stored at −80◦C until use. Real time one step RT-PCR was performed by iCycler instrument (BIO-RAD, Hercules, CA, U.S.A.) using a SYBR Green I RT-PCR quantitative kit (QBIOgene, Inc.).

A master mix of 20 μL containing 1 μL dilueted RNA, 0.1 μL AMV-RT, 4 μL SYBR Green I PCR Master Mix, 1 μL forward primer (18 pM/μL), 1 μL reverse primer (18 pM/μL), and 12.9 μL water was prepared to perform real-time RT-PCR. The forward and reverse primers for L-FABP and β-Actin cDNA were derived from a previous study [19]. Primer sequences are shown in Table II. The primers span exon/exon boundaries on the mRNA and did not amplify genomic DNA.

Reverse transcription was carried out at 55◦C for 15 min. The reverse transcriptase enzyme was heat inactivated at 95◦C for 4 min. The incubation temperature and duration of each cycle of the PCR were 30 sec at 95◦C for denaturation, 10 sec at 63-59◦C for annealing as a touchdown program and 10 sec at 72◦C for extension. The amplification was carried out for 40 cycles.

Real time quantification was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green dye to double-stranded DNA at the end of each amplification cycle. At the end of the PCR, dissociation was performed by slowly heating the samples from 55 to 95◦C and continuous recording of the decrease in SYBR Green fluorescence resulting from the dissociation of double-stranded DNA. The threshold cycle (Ct), defined as the cycle at which an increase in fluorescence above a defined baseline can be first detected, was determined for each sample. L-FABP mRNA levels were estimated on the basis of PCR efficiency and Ct deviation of an unknown sample versus a control according to 2-ΔΔCT method [20]. The β-Actin RNA was chosen as a reference gene. For validation of the method cDNA was synthesized from 10-fold serially diluted RNA samples and amplified by real time PCR using target gene specific primers. Each PCR run included a no-template control and replicates of control and unknown samples. Runs were performed in tetra plicate.

The birds fed ad libitum with dietary U/S=3.5 chosen as calibrator treatment. Using the 2-ΔΔCT method, the data were presented as the fold change in L-FABP gene expression normalized to the β-Actin gene (endogenous control) and relative to the calibrator treatment. For the calibrator control sample, ΔΔCT equals zero and 20 equals one, so the fold change in gene expression relative in calibrator control equals one, by definition. For the treated samples, evaluation of 2-ΔΔCT indicates the fold change in gene expression relative to the calibrator control. Data are presented as means ±S.D. The statistical significance is determined using the Duncans multiple range method following analysis of variance (ANOVA).

III. Results

Melting curve analysis demonstrated that each of the primer pairs described (Table II) amplified a single predominant product with a distinct melting temperature. The Tm of L-FABP cDNA product can be seen clearly as a peak in a first derivative plot. The rapid fall of at 85.5◦C indicates the presence of a specific product of L-FABP cDNA which melts at this temperature. In a pre-test, the real-time amplified RT-PCR product was sequenced (MWGBiotech, Ebersberg, Germany) and 100% homology to the chicken L-FABP sequence could be confirmed.

The Tm for β-Actin cDNA product was 89◦C. Agarose gel electrophoresis demonstrates that the L-FABP product is a single band of the predicted size, 202 bp.

Table III illustrates the effects of different dietary ratios of unsaturated to saturated fatty acids and skip a day feeding on L-FABP mRNA relative expression. At 28 and 42 days of age, no interaction found between dietary unsaturated to saturated fatty acid ratio and feed restriction. At 28 days of age, feed limitation significantly increased L-FABP mRNA expression ($P<0.01$), but after re-feeding (42 days of age), no difference found in this

respect. Dietary unsaturated to saturated fatty acids ratio of 6.5 cased an increase in L-FABP mRNA expression at both 28 and 42 days of age (P<0.05), but the changes did not follow a dose dependent manner.

TABLE I. COMPOSITION AND CALCULATED NUTRIENT CONTENTS OF BROILER GROWER (FED 10 TO 28 DAYS) AND FINISHER (FED 29 TO 42 DAYS) DIETS WITH DIFFERENT UNSATURATED TO SATURATED FATTY ACID RATIOS

ingredients	unsaturated to saturated fatty acid ratio							
	2		3.5		5		6.5	
	grower	finisher	grower	finisher	grower	Finisher	grower	finisher
Corn (g/kg DM)	42.83	49.29	46.02	52.42	47.29	53.66	47.97	54.33
Soybean meal (g/kg DM)	42.38	36.35	41.75	35.72	41.5	35.48	41.38	35.34
Sunflower meal (g/kg DM)	1.49	1.32	4.36	4.14	5.5	5.26	6.12	5.86
Beef Tallow (g/kg DM)	9.07	8.85	3.64	3.52	1.48	1.4	0.33	0.27
CaCO3(g/kg DM)	0.99	1	1	1.01	1	1.01	1	1.01
Dicalciumphosphate (g/kg DM)	1.84	1.88	1.83	1.87	1.83	1.87	1.83	1.87
Common salt (g/kg DM)	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Vitamin and MIneral premix (g/kg DM) *	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL- Methionine (g/kg DM)	0.34	0.31	0.34	0.3	0.34	0.3	0.33	0.3
HCl-Lysine (g/kg DM)	0.18	0.13	0.18	0.14	0.19	0.15	0.19	0.15
ME (kcal/kg DM)	3150	3200	3150	3200	3150	3200	3150	3200
Crud Protein (g/kg DM)	21	19	21	19	21	19	21	19
Calcium (g/kg DM)	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
Av. Phosphorus (g/kg DM)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Sodium (g/kg DM)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Lysine (g/kg DM)	1.2	1.05	1.2	1.05	1.2	1.05	1.2	1.05
Methionine (g/kg DM)	0.54	0.49	0.54	0.49	0.54	0.49	0.54	0.49
Met + Cys (%)	0.85	0.78	0.85	0.78	0.85	0.78	0.85	0.78

* Provided per kilogram: vitamin A, 4,000,000 U; cholecalciferol 800,000 U; vitamin E, 14,000 U; vitamin K3, 760 mg; vitamin B2, 2800 mg; vitamin B6, 1520 mg; vitamin B12, 7.6 mg; nicotinic acid, 18,000 mg; folic acid, 560 mg; pantothenic acid, 4400 mg; choline chloride, 19,000 mg; biotin, 45.3 mg; zinc, 16,000 mg; manganese, 25,600 mg; iron, 12,800 mg; copper, 3200 mg; selenium, 64 mg; iodine, 320 mg.

TABLE II. PRIMER SEQUENCES AND PCR PRODUCT SIZES OF L-FABP AND β-ACTIN GENES

Gene	Primer sequences (5`→3`) Forward and reverse	GenBank accession number	Product base pair
L-FABP	GAGCTCCAGTCCCATGAAAA TCAGCAGCTCCATCTCACAC	AF380999	202
β-Actin	TGCGTGACATCAAGGAGAAG TGCCAGGGTACATTGTGGTA	L08165	300

TABLE III. EFFECTS OF FEED RESTRICTION AND DIETARY FAT SATURATION DEGREE ON L-FABP RELATIVE MRNA EXPRESSION[1]

Dietary U/S **Feed Restriction**	**2** **-**	**2** **+**	**3.5** **-**	**3.5** **+**	**5** **-**	**5** **+**	**6.5** **-**	**6.5** **+**
L-FABP mRNA Expression at 28 days of age	1 ± 0.2^{d}	3.89 ± 0.08^{ab}	1.75 ± 0.15^{d}	3.78 ± 0.24^{ab}	1.83 ± 0.08^{cd}	3.07 ± 0.18^{bc}	3.61 ± 0.32^{ab}	4.65 ± 0.2^{a}
L-FABP mRNA Expression at 42 days of age	1 ± 0.28^{c}	1.42 ± 0.23^{b}	1.62 ± 0.4^{b}	2.32 ± 0.34^{a}	$1.85 \pm 0.23^{a\,b}$	1.54 ± 0.37^{b}	1.58 ± 0.18^{b}	2.48 ± 0.1^{a}
	Probability> F							
Analysis of Variance	U/S	FR	U/S × FR					
L-FABP mRNA Expression at 28 days of age	*	**	NS					
L-FABP mRNA Expression at 42 days of age	*	*	NS					

**P< 0 01; *P < 0 05. U/S: Dietary Unsaturated to Saturated Fatty Acids Ratio; FR: Feed Restriction as skip a day feeding at 10-28 Days of age. a–d Values in the same column in each comparison group, with no common superscript differ significantly; P <0.05. [1]Values are means of four determinations of L-FABP relative gene expression (4 birds per age / each feeding program / each U/S Ratio).

IV. Discussion

Generally, tissues with an active fatty acid metabolism, such as liver, express high levels of FABPs that harmonize fatty acid absorption and utilization. The liver has a major role in fat metabolism, and considerable amounts of fatty acids are transferred into and out of this organ.

Evidence imply that L-FABP does not undergo major modulation with the changes from the fed to fasted to re-fed states, during which rapid, considerable transitions in hepatic fatty acid flux, fatty acid oxidation, and triglyceride biosynthesis take place [16]. Starvation activates comprehensive adaptative metabolic reactions including energy-metabolic responses, a route involves tissue specific changes in gene expression and in which the liver plays an essential role.

In the current study, a durable feed restriction caused a temporary and notable increase in L-FABP gene expression. There are same reports too. In the study of Ref [15], feed restriction and re-feeding did not result in unidirectional alterations in L-FABP gene expression; a 4 hours feed restriction to some extent reduced the LFABP mRNA level, while feed restriction for 20 hours caused a 2.2-fold increase in L-FABP mRNA level compared with that of the fed group. Moreover, re-feeding after feed restriction had an irrelevant effect on the L-FABP mRNA expression, so that these authors suggested that feeding motivation may not be a major factor regulating L-FABP gene expression.

The effect of feed restriction on L-FABP gene expression enhancement is in agreement with the findings of Ref [19] in broiler breeder hens. In their report, feed restriction resulted in a decrease in expression of genes related to lipogenesis but an increase in expression of genes like L-FABP that involve in lipids transportation.

It seems that during lipolysis following feed restriction, body has to transfer more amounts of fat hydrolysis products, and this can lead to an increase in FABPs production. This is in agreement with ref [8] that showed a positive correlation between FABPs gene expression and cellular fatty acids metabolism. It is assumed that the stimulation of L-FABP gene expression is activated by the raise in NEFA influx into the liver [15].

Ref [21] found no significant difference in L-FABP gene expression by dietary fat saturation degree. But in our research, dietary unsaturated to saturated fatty acid ratio of 6.5 interestingly raised L-FABP gene expression. In present study, no significant interaction between type of feeding and dietary fat unsaturation degree indicates that these factors affect L-FABP gene expression independently.

The higher expression of the L-FABP gene in the livers of the broilers following feed deprivation and high unsaturated fatty acids ratio might result in increased fatty acids transportation into mitochondria or peroxisomes and enhanced fatty acids oxidation and reduced depot fat. So the regulation of L-FABP Gene expression could have an effect on fat deposition in the chicken.

Present study provides evidences to the physiological functions of L-FABP in the avian liver, however, There are reports on the effects of other factors on the triggering of L-FABP too. For example, it has been obviously showed that growth hormone increase L-FABP gene expression in the liver of rats [22]. This observation proposes that birds have a mechanism for regulation of fatty acids transfer under different nutritional condition, but it seems that, further studies will be needed to explain the exact mechanism for the increase in L-FABP gene expression.

References

[1] B. M. Freeman, *Physiology and Biochemistry of the Domestic Fowl*, Academic Press, London, 1984, vol. 5.

[2] H. W. Hulan, F. G. Proudfoot, and D. M. Nash, "The effects of different dietary fat sources on general performance and carcass fatty acid composition broiler chickens," *Poult. Sci.*, vol. 63, no. 2, pp. 324-332. Feb. 1984.

[3] M. W. Yu and F. E. Robinson, "The application of short-term feed restriction to broilers chickens production: A review," *J. Appl. Poult. Res.*, vol. 1, no. 1, pp. 147-153, Mar. 1992.

[4] J. F. Glatz and G. J. Vander Vusse, "Cellular fatty acid-binding proteins: their function and physiological significance," *Prog. Lipid Res*., vol. 35, no. 2, pp. 243–282, Sep. 1996.

[5] N. R. Coe and D. A. Bernlohr, "Physiological properties and functions of intracellular fatty acid-binding proteins," *Biochem. Biophys. Acta—Proteins Proteomics*., vol. 1391, no. 3, pp. 287–306, Apr. 1998.

[6] J. Storch and B. Corsico, "The emerging functions and mechanisms of mammalian fatty acid-binding proteins," *Annu. Rev. Nut*., vol. 28, no. 1, pp. 73–95, Aug. 2008.

[7] J. Storch and A. E. A. Thumser, "The fatty acid transport functions of fatty acid-binding proteins," *Biochem. Biophys. Acta—Proteins Proteomics*., vol. 1486, no. 1, pp. 28-44, Jun 2000.

[8] R. A. Weisiger, "Cytoplasmic transport of lipids: Role of binding proteins," *Comp. Biochem. Physiol*, vol. 115, no. 3, pp. 319–331, Nov 1996.

[9] N. H. Haunerland and F. Spener, "Fatty acid-binding proteins–insights from genetic manipulations," *Prog. Lipid Res*, vol. 43, no. 4, pp. 328–349, July 2004.

[10] D. R. Prows, E. J. Murphy, and F. Schroeder, "Intestinal and liver fatty acid binding proteins differentially affect fatty acid uptake and esterification in L-cells," *Lipids*, vol. 30, no. 10, pp. 907–910, Oct. 1995.

[11] E. P. Newberry, Y. Xie, S. Kennedy, X. Han, K. K. Buhman, *et al*., "Decreased hepatic triglyceride accumulation and altered fatty acid uptake in mice with deletion of the liver fatty acid-binding protein gene," *J. Biol. Biochem*, vol. 278, no. 1, pp. 51664–51672, Jan 2003.

[12] N. J. Spann, S. Kang, A. C. Li, A. Z. Chen, E. P. Newberry, *et al*., "Coordinate transcriptional repression of liver fatty acid-binding protein and microsomal triglyceride transfer protein blocks hepatic very low density lipoprotein secretion without hepatosteatosis," *J. Biol. Chem*, vol. 281, no. 1, pp. 33066–33077, Jan. 2006.

[13] C. Wolfrum, C. M. Borrmann, T. Börchers, and F. Spener, "Fatty acids and hypolipidemic drugs regulate peroxisome proliferator-activated receptors α- and γ- mediated gene expression via liver fatty acid binding protein: A signaling path to the nucleus," *Proc. Nat. Acad. Sci. USA*, vol. 98, no. 23, pp. 2323–2328, Oct. 2001.

[14] S. M. DiPietro, J. H. Veerkamp, and J. A. Santome, "Isolation, amino acid sequence determination and binding properties of two fatty-acid-binding proteins from axolotl (Ambistoma mexicanum) liver–evolutionary relationship," *Eur. J. Biochem*, vol. 259, no. 1, pp. 127–134. Jan. 1999.

[15] A. Murai, M. Furuse, K. Kitaguchi, K. Kusumoto, Y. Nakanishi, M. Kobayashi, and F. Horio, "Characterization of critical factors influencing gene expression of two types of fatty acid-binding proteins (L-FABP and Lb-FABP) in the liver of birds," *Comp. Biochem. Physiol, Part A*, vol. 154, no. 2, pp. 216–223, Oct. 2009.

[16] N. M. Bass, "The cellular fatty acid binding proteins: aspects of structure, regulation and function," *Int. Rev. Cytology*, vol. 111, no. 1, pp. 143–184, Jan. 1988.
[17] J. H. Veerkamp, "Fatty acid transport and fatty acid-binding proteins," *Proc. Nut. Soc*, vol. 54, no. 1, pp. 23–37, Mar. 1995.
[18] J. Huang, D. Yang, S. Gao, and T. Wang, "Effects of soy-lecithin on lipid metabolism and hepatic expression of lipogenic genes in broiler chickens," *Livestock. Sci*, vol. 118, no. 1, pp. 53–60. Oct. 2008.
[19] M. P. Richards, S. M. Poch, C. N. Coon, R. W. Rosebrough, C. M. Ashwell, and J. P. McMurtry, "Feed Restriction Significantly Alters Lipogenic Gene Expression in Broiler Breeder Chickens," *J. Nut*, vol. 133, no. 3, pp. 707–715, Mar. 2003.
[20] K. J. Livak and T. D. Schmittengen, "Methods. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T))," *Methods*, vol. 25, no. 4, pp. 402–408, Dec. 2001.
[21] E. Duplus, M. Glorian, and C. Foresti, "Fatty acid regulation of gene transcription," *J. Biol. Biochem*, vol. 275, no. 40, pp. 30749–30752. Oct. 2000.
[22] L. Carlsson, I. Nilsson, and J. Oscarsson, "Hormonal regulation of liver fatty acid binding protein in vivo and in vitro: Effects of growth hormone and insulin," *Endocrinol*, vol. 139, no. 6, pp. 2699–2709, Jun. 1998.

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