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University of Dayton

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**STUDIES OF FATTY ACID BINDING PROTEIN IN RAT LIVER AND THE
ISOLATION, PURIFICATION, CHARACTERIZATION OF FABP
FROM PORK LIVER**

Thesis

Submitted to

The College of Arts and Sciences of the
UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Master of Science in Chemistry

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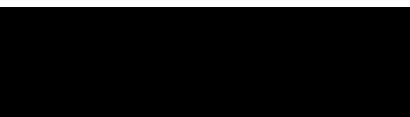
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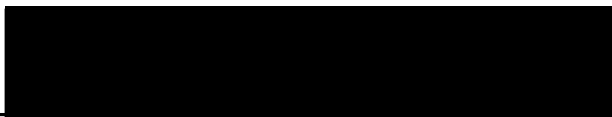
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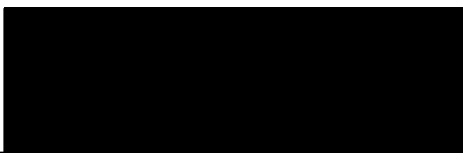
APPROVED BY:



Sanford S. Singer, Ph.D. Professor, Committee Chair



Kevin M. Church, Ph.D., Professor, Committee Member



Vladimir A Benin, Ph.D. Professor, Committee Member

ABSTRACT

STUDIES OF FATTY ACID BINDING PROTEIN IN RAT LIVER AND THE ISOLATION, PURIFICATION, CHARACTERIZATION OF FABP FROM PORK LIVER

Name: Durga Soujanya Varre

University of Dayton

Advisor: Dr. Sanford S Singer

The objectives of the research were to study the effects of cardiovascular drugs on LFABP, IFABP and LFABP/IFABP ratio and developing FABP isolation from pork liver for biosensor use. For this I used SHR and Holtzman rat tissues and supermarket pork liver to explore fatty acids and FABP level, and tested cardiovascular drugs. DEAE Sephadex G-75 chromatography was performed. Assays used rose Bengal in our standard method. Pork liver FABP preparation used ammonium sulfate fractionation, DEAE Sephadex A-50, Sephadex G-75& and Sephadex G-50 chromatography. Rose Bengal assay, Biuret assay and conductivity tests were done. The characterization involved chromatography, gel electrophoresis, molecular weight determination, radioactivity tests and isoelectric focusing.

Results showed an increase in [LFABP]/g tissue and per 100 g body weight after administering the drugs. IFABP did not increase significantly. A significant increase in LFABP/IFABP per 100 g body weight also occurred. The blood pressures in the treated SHR rats decreased significantly, as expected.

Total purification achieved in pork liver was 270 fold. The specific activity increased between each step. The mean molecular weight of the FABP was

15.6 \pm 4.5 kDa. A significant amount of purified FABP was isolated. However, the FABP sample was not homogenous since two types of FABP were present.

The work helped us to understand the alteration of LFABP and IFABP levels and the changes in their ratios in drug fed rats: to explain their action in cardiovascular disease; expand our ability to study a rat-dog model for FABP action and to develop a method of isolation of FABP from super market pork liver. This FABP is inexpensive compared to FABP isolated from fresh rat tissue. This in time could provide capability for making biosensors for disease diagnosis.

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I cannot end without thanking my brothers Vijay Bhanu Varre, Anil Kumar Varre, my sisters-in-law Dr. Sowmya Varre & Dr. Rekha Madhuri on whose constant encouragement and love I have relied throughout my time at the University. Words fail me to express my appreciation to my husband Dr. Sunil Musinipally whose dedication, love and persistent confidence in me, has taken the load off my shoulder.

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LIST OF ABBREVIATIONS

| | |
|--------------------------------------|---|
| ALBP..... | Adipocyte lipid binding protein |
| AMS..... | Ammonium sulfate |
| BSA | Bovine serum albumin |
| C..... | Centigrade |
| CPM..... | Counts per minute |
| cm..... | Centimeter |
| CMC..... | Carboxymethylcellulose |
| cNMR..... | Carbon 13 nuclear magnetic resonance |
| CRBP..... | Cellular retinoic acid binding protein |
| CH..... | Chymotrypsin |
| CONT..... | Control |
| CY..... | Cytochrome-c |
| DEAE..... | Diethylaminoethyl |
| Da..... | Dalton |
| DI-water..... | Deionized water |
| EPS 600..... | Electrophoresis power supply |
| ETOH..... | Ethyl alcohol |
| EXPT..... | Experimental |
| DM..... | Direct method or spectrofluorimetric method |
| FABPs..... | Fatty-acid-binding proteins |
| FA..... | Fatty acids |
| HDL..... | High density lipoprotein |
| HoRs..... | Holtzman normotensive rats |
| HPLC..... | High performance liquid chromatography |
| H ₃ PO ₄ | Phosphoric acid |
| HFABP..... | Muscle and heart FABP |
| IACUC..... | Institutional Animal Care and Use Committee |
| IEF..... | Iso electric focusing |
| IFABP..... | Intestinal FABP |
| IHM..... | In House Method |
| kDa..... | Kilo Dalton |

| | |
|--|---|
| LDL..... | Low density lipo protein |
| LFABP..... | Liver FABP |
| MDGI..... | Mammary derived growth inhibitor |
| mg..... | Milligram |
| mL..... | Milliliter |
| M..... | Molar |
| min..... | Minute |
| Mm..... | Millimolar |
| MW..... | Molecular weight |
| MY..... | Myoglobin |
| Na ₂ HPO ₄ | Sodium phosphate |
| NaCl..... | Sodium chloride |
| nm..... | Nanometer |
| NEN..... | New England Nuclear Corp |
| NMR..... | Nuclear magnetic resonance |
| OV..... | Ovalbumin |
| P..... | Probability |
| RBu..... | Red binding unit |
| SA..... | Specific activity |
| SEM..... | Standard error of the mean |
| SHR..... | Spontaneously hypertensive rats |
| TSM..... | 0.050M Tris, 0.25M sucrose, 3.00mM 2-Mercaptoethanol (pH = 7.5) |
| Ve..... | Evolution volume |
| Vo..... | Void volume |

CHAPTER I

INTRODUCTION

The uptake and biosynthesis of water soluble and insoluble metabolites is essential for every living cell. Fatty-acid-binding proteins (FABPs) are a small group of highly conserved carrier proteins thought to be involved in the uptake, transport and metabolism of fatty acids (FAs) and other lipophilic substances such as eicosanoids and retinoids, between extra- and intracellular membranes similar to the extra cellular function of albumin [1,2]. Some FABPs are also believed to transport lipophilic molecules from outer cell membrane to certain intracellular receptors such as PPAR [3]. They may also be responsible in the modulation of cell growth and proliferation.

FABPs are found in the cytosol of many tissues that utilize and/or transport long chain FAs. In several different circumstances involving nutritional, hormonal and pharmacologic manipulations, a significant correlation can be demonstrated between cytosolic FABP and rates of fatty acid influx into the cell [4]. These proteins bind to hydrophobic ligands. The abundance of hepatic and intestinal FABPs is subject to hormonal and dietary control [5, 6].

FABPs are mainly concentrated around organelles where β oxidation occurs. Also, the activity of FABPs appears to be highly correlated with the capacity of tissues to synthesize lipids in vitro [7]. Thus it is important to understand the factors that regulate the level and activity of these proteins [8]. The structures of 3 families of proteins which bind FAs, albumin, lipocains and FABPs have been described [9]. Many of the proteins of this family bind FAs as their main ligand, but other proteins with quite different structure also have affinity for FAs. The FABPs form part of the super family of the calicyns with a β barrel structure [9-12].

The intracellular fatty acid-binding proteins (FABPs) belong to a multigene family with nearly twenty identified members. The existences of FABPs were reported by Ockner, Mannig, et al. in intestinal mucosa [5]. Immunochemical analysis based on reactivity to different antibodies indicated the presence of 9 different kinds of FABPs which are not identical and are differentiated based on reactivity to different antibodies. The 9 types of FABP include liver FABP (LFABP), intestinal FABP (IFABP), muscle and heart FABP (HFABP), adipocyte lipid binding protein (ALBP), mammary derived growth inhibitor (MDGI), myelin P2, cellular retinoic acid binding protein (CRBP 1, CRBP 2). FABPs are divided into at least three distinct types, namely the hepatic-, intestinal- and cardiac-types. They are 14-15 kDa proteins [11].

Liver, central to fatty acid metabolism, responds to injury by fat accumulation causing other health problems. Liver fatty acid transport is related to understanding lipid disease and LFABP is likely to be important. It is seen to transport fatty acids between cell organelles. It also binds 60 % of liver cytosol fatty acids and controls lipid metabolism enzymes. Because LFABP levels and transport capacity change greatly in early fatty liver development, it is seen as rate limiting for liver lipid metabolism and related processes. This is shown by LFABP level elevation by hypolipidemic clofibrate, accompanied by increased fatty acid uptake. Also it is observed that LFABP levels drop in old rats. Fasting drops LFABP levels too, and its levels are 50% higher in adult females than males. This may relate gender differences in tissue fat levels and hepatic fatty acid transport/ metabolism. FABP levels were found to drop on aging [4].

The significance of FABPs in lipid metabolism and lipid related diseases has been a topic of research for over 20 years. In recent years the role of FABPs in insulin sensitivity in connection with intramuscular lipid content, type 2 diabetes, obesity, mediating the effects of 2 classes of peroxisome proliferators and cytoplasmic transport of hydrophobic molecules have been explored by various scientists. In vitro and whole

animal studies support roles of FABPs in beta-oxidation of FAs and bioeffects of cardiovascular drugs like tiadenol and fenofibrate.

All FABPs have similar molecular masses and structures but amino acid sequences vary. FABP content in various organs and organisms differ and may be related to the ability to metabolize/transport lipids. The differences support roles in heart disease and species nutrition capacity. Relationships of FABPs to heart disease diabetes and high body lipid content in aging are probable. In heart disease, treatment with hypolipidemic drugs (e.g. clofibrate) may be due to raise LFABP levels. Furthermore, altered IFABPS are related to human diabetes [6].

The intracellular or cytoplasmic FABPs are 14 to 15 KDa proteins of 126 -134 amino acids, and are named after the first tissue of isolation or identification. Some tissues contain several types, either in different cell types (brain, kidney, stomach) or in the same cell type (enterocyte). LFABP and IFABP bind more bulky hydrophobic ligands such as lysophospholipids, prostaglandins, bile acids eicosanoids and some drugs [13-15].

The pattern of tissue expression of FABP found in many non-mammalian tissues was different from mammals. FABPs from the same mammalian tissue of different species show greater amino acid identity than those isolated from different tissues of the same species. The hypothesis that FABPs have developed distinct binding sites in order to perform specific functions within the different tissues in which they are expressed was supported by molecular information [16, 17, and 18].

Structure of FABP

The tertiary structure of FABPs has been revealed by crystallography and/or nuclear magnetic resonance (NMR) [19- 23]. The structural features of all FABPs are similar and are composed of ten anti parallel β -strands that form a β -barrel. The bound ligand is found within the barrel in a central internal water filled cavity. The interior of the cavity is determined by the side chains of both hydrophobic and polar amino acids, and is variable between the different FABP types. The buried amino acids are assumed to contribute to protein stability.

Certain internal water molecules are well ordered and highly conserved in homologous proteins. In FABPs, these water molecules function in the displacement of FA and maintain the electrostatic interactions inside the binding cavity. The decreased number of intra-main chain hydrogen bonds girding the portal region suggests a greater degree of possible motion in L-FABP. All FABP structures determined to date showed α -helical regions. According to fatty acid transfer studies effective collisional transfer of FA to phospholipids membranes does not occur in the absence α -helical domain, thus showing that α -helical region is essential for interaction with membrane [24, 25].

Functions of FABP

The structural-functional relationship of FABPs has been studied by site-directed mutagenesis. Various single amino acid mutants of different FABP types have been studied for ligand specificity and affinity and protein stability [26].

Fatty acid uptake and transport

Most eukaryotic cells were found to be capable of taking up long chain FAs to be used for a variety of cellular processes. In eukaryotic cell FA uptake occurs by both passive diffusion and by protein mediated binding and translocation mechanisms. Diffusion of FAs across the plasma membrane is a fast process, also called 'flip-flop'. The process is driven by an inwardly directed FA gradient, with an extracellular FA concentration in 10-20 fold excess over the intracellular content [27-29]. Hamilton and Kamp suggested plasma membrane FA transporters function indirectly by increasing the FA partitioning into the membrane-bound enzyme, thereby enhancing metabolism [30]. According to Stump et al. the rate constants for saturable transmembrane influx were larger than those for non-saturable uptake [31]. FAs are bound by FABPs intracellularly. FABPs are considered to be important carriers for intracellular FAs. Several studies have demonstrated FA transport between FABPs and membranes by use of radiolabelled or fluorescent FAs or by NMR. The transfer rate differs significantly between different FABPs: A-FABP > H-FABP > I- FABP > L- FABP [32].

Modulation of signal transduction and gene transcription by FABPs

Various cellular processes may be influenced by FAs, their CoA, carnitine esters and other lipid modulators like eicosanoids and lysophospholipids, by interactions with enzymes, membranes, ion channels, receptors or genes [13, 33-35]. FABPs have an indirect effect on these processes by modulation of the concentrations of unesterified FAs. L- FABP binds to FAs, lysophospholipids and eicosanoids. As the concentrations of FAs are rapidly changed in response to binding of specific agonists to plasma

membrane receptors they are able to act as second messengers involved in signal transduction.

Growth factor induced diacylglycerol kinase α -activation in vascular smooth muscle cells is inhibited by FAs. Increased levels of FAs may contribute to chronic protein kinase C activation with diabetes. Polyunsaturated FAs directly regulate Na^+ , K^+ , Ca^{2+} and Cl^- ion channels [36-40]. FAs play an important role in transcription of genes, especially those genes which encode proteins involved in lipid metabolism. The transcription of FABP and FATP genes is also promoted by FAs. FABPs have been detected in the nucleus of hepatocytes, heart myocytes, locust myocytes, and astrocytes. Data from fluorescein conjugated FABP showed that L- FABP [41] is involved in communicating the state of FA metabolism from cytosol to the nucleus through an interaction with lipid mediators that are involved in nuclear signal transduction [42].

The physiological role of FABP was understood by studies on knockout mice. The loss of I- FABP resulted in normal phenotype, hyperinsulinemia and gender specific body weight gain, while the loss of L- FABP resulted in normal phenotype and normal bile acid pool size. The loss of H- FABP resulted in exercise intolerance, localized cardiac hypertrophy, reduced oleate /palmitate uptake and increased glucose oxidation [43, 44].

Role of FABP in growth, differentiation and cytoprotection

Cell differentiation and postnatal development for various FABPs in different tissues is responsible for the expression of FABP mRNA and FABP synthesis [16, 17, and 45]. FABP also seems to be responsible for modulation of cell growth. L- FABP modulates the mitogenesis of liver and hepatoma cells leading to a higher proliferation

rate and multiplication of hematoma cells. From these findings it appears that L- FABP may play an important role in regulating embryonic cell differentiation by acting in the nucleus as well as the cytoplasm.

FABPs could have been used as tumor markers. Changes in FABP content seems to be connected with the development of liver and gut cancer and the level of L FABP and I- FABP decreased in rat and human colon carcinoma [46, 47] . It was also found that L- FABP expression is suitable for use as a new pre-surgical prognostic marker patients undergoing hepatic surgery for colorectal cancer metastases [48].

Role of FABP in diagnosis of tissue damage

The small size, high solubility and tissue specificity make FABPs and their serum concentrations good candidates as biochemical markers for tissue injury providing information about the nature and extent of tissue damage. It was found that FABP released after acute myocardial infarction is quantitatively recovered in plasma, making it a useful biochemical plasma marker for the estimation of infarct size in humans [49, 50]. Similarly, L- FABP was found to be released from the liver in various liver diseases and I- FABP into the circulation in acute phase of intestinal ischemia making it a potential marker to facilitate early detection of mesenteric ischemia. Some studies have also shown that I- FABP could be used as a serum marker in detecting acute intestinal allograft rejection, although it was contested by others [51].

Basis for work on rats

Cytosolic LFABP and IFABP take part in FA transport from the intestine to blood to the liver for bioenergy production and cell component biosynthesis. Therefore the genetic malfunction of either FABP or variation of the ratio of LFABP to IFABP content

(LFABP/IFABP) may cause or be associated with altered lipid uptake metabolism and lipid related diseases [54-57].

We found that significant differences of LFABP and IFABP levels and LFABP/IFABP ratio occur in Okamoto, spontaneously hypertensive rats and their normotensive controls of both genders. This suggested the probability of elevated blood lipid levels that could lead to cardiovascular complications such as atherosclerosis and hypertension [58-59]. It was also observed in our lab that statistically significant differences of LFABP and IFABP levels and increase of LFABP/IFABP in Holtzman rats (both genders) fed clofibrate fit in with the hypolipidemic effects of the drug and may account for clofibrate action in preventing and reversing cardiovascular problems. Therefore we were interested in looking at effects of drugs used in treating cardiovascular disease on FABP levels in liver and intestine.

The routine method our group uses for Sephadex G-75 chromatography followed by rose Bengal assay is very complex, labor intensive and daylong [60-62]. So, it would be helpful to devise a method that allows direct assay of rat FABP in cytosol that can be done in 1 to 2 hours. The spectofluorimetric method (DM) developed by Mridul Bhatula was successful in achieving this. Furthermore, rat liver is used for making the FABP used for these experiments [63]. To buy purified rat FABP is very expensive (e.g., 1 μ g pure FABP costs \$273). The size of rat liver is also very small; so many rats are killed to make FABP by our in-house method which provides us with FABP for study at a lower price. Preliminary data with beef liver by Brandon Dreyer [64], and chicken liver by RaviChand Yelamanchili show that such inexpensive tissue, available at grocery stores, may serve this purpose.

CHAPTER II

OBJECTIVES OF RESEARCH

- 1) To study the effects of clofibrate and fenofibrate, drugs used in treating cardiovascular disease, on the FABP levels in rat liver and intestine; as well as the LFABP/IFABP ratio and identify whether drops in blood pressure and elevated LFABP/IFABP ratios are similar to data we took with younger SHR_s.
- 2) To compare reported anti-cardiovascular potency of clofibrate with fenofibrate potency by examining LFABP/IFABP ratios in male rats given the drugs.
- 3) To develop the method of isolation of FABP from frozen pork liver obtained from supermarkets.

CHAPTER III

MATERIALS AND METHODS

Animals

Male Holtzman Sprague-Dawley rats (HoR), and male spontaneously hypertensive rats (SHR), were purchased from Harlan Sprague-Dawley (Indianapolis, IN). They were maintained in the University of Dayton Vivarium on Formula diet # 5008 (PMI Nutrition International, Brentwood, MO) and tap water, ad libitum, until used. This study was carried out in collaboration with Matthew Boka whose results also went into his Undergraduate Senior Research Thesis [67]. We used 44 male rats, divided into 6 groups of ~ 7 animals, of which 14 male SHRs were controls, 8 male SHRs were fed with clofibrate and 6 male SHRs were injected with fenofibrate. The remaining 16 were Holtzmans. They included 7 male controls and 9 males injected with fenofibrate.

The SHRs given control and experimental diets were 3.5 to 5 months old. They were fed ad libitum, every other day and were used 2.5 to 3 weeks after start of diet. The clofibrate diet was from Harlan Sprague-Dawley (Madison, WI). The intubated Holtzman rats were 4.5 to 5 months old. They were intubated every other day and were used 2 to 3 weeks after intubation was started. The fenofibrate injection (Sigma, St. Louis, MO) consisted of 0.2% of the drug in 1% carboxymethylcellulose (CMC, 25mg/mL). The controls for this group were given Formula Diet #5008 (PMI Nutrition International and, saline CMC Brentwood, MO). The University of Dayton's IACUC approved use of the animals for this work in protocols on file in the office of the Vice President for Graduate studies and Research and in the IACUC records.

Pork Liver Tissue

Eight different samples of pork liver (~4 pounds each) were obtained from local supermarkets (Meijer's and Kroger's) and were stored frozen at -15°C for 0 to 2 months before use. The plan to use this tissue arose from the work of Brandon Dreyer in our group [64], which indicated that frozen beef samples sold for consumption worked like fresh beef livers. Pork liver is softer than beef liver. So we hoped to achieve better yields than achieved with beef tissue by reducing the difficulty of mechanical disruption of harder tissue that might have led to heating effects causing inactivation of FABP.

Chemicals

The cardiovascular drugs clofibrate® (Atromid) and fenofibrate® (Tricor), bovine serum albumin, chymotrypsin, horse myoglobin, ovalbumin, and cytochrome-c, Dextran, charcoal, rose Bengal, enzyme grade sucrose, KCl and Tris base (trishydroxymethylaminomethane)., were bought from Sigma-Aldrich, (St. Louis, MO). DEAE Sephadex A-50, both Sephadexes G-50 and G-75, Blue Dextran 2000, Phast gradient gels (10-15% gels), Phast gel blue R, and buffer strips were all purchased from Pharmacia-LKB (Piscataway, NJ), [^3H]Palmitate and [^3H] Oleate (35.6 Ci/mmol) came from New England Nuclear Corp., (Boston, MA) , Methanol, acetic acid, glycerol, and sodium phosphate were products of Sigma-Aldrich (St Louis, MO). Biosafe-2 Liquid scintillation cocktail was obtained from Research Products International (Mount Prospect, IL) .All other chemicals and supplies were obtained in highest quality from standard suppliers.

Experiments on Rats

Cytosol preparation

Rats were sacrificed by decapitation as approved by the University of Dayton IUCAC. Blood pressures of each of the rats were taken on the night before sacrifice [60]. Their livers and intestines were quickly removed, weighed and chilled on ice. From 4 to 5 g of liver (1 to 2 g portions were cut from each lobe), trimmed into smaller pieces, were used from each rat. The intestines were each divided in half and the first (proximal) halves were trimmed free of adipose tissue, cleaned with 1% saline, and weighed.

All preparative procedures from this point on were carried out at ice bucket temperature. Each liver and intestine sample was homogenized [60] separately in a Potter-Elvehjem homogenizer [62]. The intestine muscle tissues were removed from homogenates, weighed, and their weights were subtracted from the weight of the intestines, to give mucosa weights.

Homogenates were prepared in 1mL/g ice cold 0.050M Tris, 0.25M sucrose, 3.00mM 2-mercaptoethanol, pH 7.5 (TSM). The homogenates were poured into centrifuge tubes and placed into a fixed angle 40 rotor (Beckman Instrument Company, Fullerton, CA). The samples were then centrifuged at $105,000 \times g$ for 60 min at $2^{\circ}C$ and 1 torr (Beckman L-565B centrifuge, Beckman Instrument Company, Fullerton, CA) within 15 minutes after preparation [62]. Finally the supernatants were drawn off and their volumes were recorded.

Sephadex G-75 chromatography

The 3-5 mL cytosol samples from each rat liver or mucosa sample were each subjected to chromatography on a column of the molecular sieve, Sephadex G-75, using

1.25x70 cm columns, filled with the sieve [60]. In every experiment, the fractionation on G-75 columns was carried out simultaneously with samples from liver and intestine. The columns were pre-equilibrated with ~500mL TSM, washed through and elution with TSM was carried out. Sixty samples (1.5 mL each, fractions) were collected using Redifrac fraction collectors (Pharmacia LKB, Piscataway, NJ).

Solutions and Chemicals

TSM buffer, YSI-2357 buffer (YSI Life Sciences, Yellow Springs, OH), Sephadex G-75 resin, rose Bengal dye, and dextran-charcoal were used. The chemicals were purchased from Sigma-Aldrich Chemical Company (Sigma-Aldrich Corp., St. Louis, MO), unless otherwise indicated. All solutions were made in deionized water unless noted otherwise.

TSM buffer contains 0.050 M Tris base, 0.25 M sucrose, and 3.00 mM 2-mercaptoethanol (neutralized to pH 7.5). It was the buffer used during all experiments, unless otherwise indicated. It was made in 1.00 L deionized water, as follows. First, 6.05 g Tris base, 85.5 g sucrose and 0.30 mL mercaptoethanol were weighed and added to 750 mL cold deionized water (DI-water). Then the mixture was stirred until the solids dissolved. The pH of the solution was measured and adjusted to pH 7.6. Then 250 mL of DI-water were added. The solution was stirred in a cold box until it was cold, and the pH was checked again and restandardized, if necessary [60].

Sephadex G-75 (Amersham Biosciences, Piscataway, NJ) was used to separate FABP from other proteins in cytosol by column chromatography. Rose Bengal solution (40.0 mM) was prepared by mixing 41.0 milligrams of rose Bengal in one liter of deionized water for the regular assay used in the In House Method (IHM). The use of the

dye in all assay methods will be described in the second section of this chapter.

Dextran-coated charcoal was prepared by mixing 0.828 g of Na_2HPO_4 (Fisher Scientific Co., Pittsburgh, PA) with 200 mL of cold (4°C) deionized water and titrating it to pH 7.4. Then, 1.0 g of Dextran T-170 (Fisher Scientific Co., Pittsburgh, PA) and 10.0 g of Norit A decolorizing carbon (Fisher Scientific Co., Pittsburgh, PA) were added to the solution. Dextran-coated charcoal was used to remove rose Bengal dye which did not bind to the protein.

Assays

Column fractions underwent three assays: a 280 nm Protein Assay to determine the location of non-FABP proteins; a 560 nm Rose Bengal [60] Assay to identify FABP-rich fractions; a 560 nm Pool Assay to identify the [LFABP], the [IFABP] and the ratio of LFABP to IFABP content (LAFABP/IFABP) per g tissue and per 100 g bodyweight.

- a) 280 nm Protein Assay: For liver studies, a 0.10 mL sample of each chosen column fraction (usually every third fraction) was mixed with 0.90 mL TSM. 280 nm absorbance readings were taken in our Genesys spectrophotometer (Spectronic Instruments, Rochester, NY) [62]. For intestine studies 0.30 mL samples, mixed with 0.70 mL TSM were used.
- b) 560 nm Rose Bengal Assay: Test samples (from every third chromatogram fraction) were mixed with enough TSM (intestine samples were 0.30 mL and liver samples were 0.10 mL. Then, 0.30 ml 40.0 mM rose Bengal was added, followed by incubation (5 min, 0°C). Next, 0.15 mL charcoal-dextran was added, followed by incubation (8 min, 0°C). Then, the tubes were centrifuged for 8 minutes using Centra B-plus centrifuges (International Equipment Co., Needham

Heights, MA) and the 560 nm readings of the supernatants were done. The 560 nm absorbance readings obtained were plotted to identify the FABP rich fraction.

- c) 560 nm Rose Bengal Pool Assay: FABP-rich fractions were identified, pooled and their volumes were measured. They were diluted if necessary (e.g., in the cases where drugs tripled the levels of FABP in liver) and 560 nm rose Bengal assay was carried out on each pool. Absorbance values observed at 560 nm were converted into mg FABP per g liver and per 100 g body weight (RBU/mL) as shown next.

Calculations

A series of calculations were used to obtain the amount of FABP in mg per g liver. First, the absorbance read for the pool assay was divided by the volume of the pool to obtain absorbance per mL.

$$\text{Absorbance / mL pool} = \frac{\text{Absorbance 560}}{\text{Pool volume in sample}}$$

Then the readings were averaged, and the total absorbance in the pool (in red binding units, RBU) was calculated by multiplying the average by 0.67, a factor devised to correct for changes in assay volumes in 1997 in our laboratory

$$\text{Total RBU} = \frac{(\text{average RBU/mL}) \times (\text{Pool Volume})}{0.67}$$

The mg of FABP per gram tissue was calculated by dividing the total red binding units by 3.69 (the product of assay correction factor 2.86 and 1.29, the absorption of 1 mg pure LFABP in the assay) and the weight of the tissue chromatographed.

$$\text{mg FABP/g liver} = \frac{\text{Total RBU}}{(3.69) \times (\text{corrected weight})}$$

Statistical significance

Statistical significance of differences between control and experimental groups ($p < 0.05$) was determined by use of student's test [60, 62].

Development of purification of pork liver FABP through Sephadex G-50 step

About 100 g of frozen pork liver was cut into small pieces, mixed with TSM (1 mL for every g) and dispersed in a Waring blender for ~1 min, followed by homogenization in a Potter-Elvehjem homogenizer for 6 passes. All methods were carried out at ice bucket temperature. Next, the homogenate was poured into centrifuge tubes and centrifuged at 12,000 rpm in a GSA rotor in a Sorval RC2B centrifuge (Dupont-Sorval, Newton, Connecticut) for 40 minutes. Then, the supernatant was drawn off and its volume was recorded.

Ammonium Sulfate (AMS) Cuts

The 12,000 rpm supernatant was subjected to consecutive AMS cuts (0-35%, 35-50%, 50-75%). This step separated pork liver FABP from pork liver in the 50-75% saturated AMS fraction. For each cut, the amount of solid AMS to be added was calculated from the AMS table [58] and depended on the volume of the supernatant. The AMS was triturated to a fine powder using a mortar and pestle. It was then added slowly over 15 min with gentle stirring in an ice bath on a magnetic stirrer. Then, the sample was allowed to stir for an additional 10 min. Next it was centrifuged in the Sorval

centrifuge (SS-34 rotor) for 30 min. After centrifugation the pellets from the 0-35% and 35-50% cut, which contained very little FABP, were discarded. The pellet from the 50-75% cut which contained the FABP (see next section) was resuspended in enough TSM to bring the volume of the resuspendate to 12-15 mL.

Sephadex G-75 chromatography

Sephadex G-75 columns {1.9 cm x 70 cm} were washed by elution with ~500 mL TSM buffer at a rate of 4 drops per min. The resuspended 35-50% and 50-75% cut material was then chromatographed on separate columns and eluted with TSM at a rate of 10 drops per min. Fractions of 120 drops each were collected on Pharmacia Redifrac fraction collectors. Following chromatography, fractions were chosen for protein {280} and pink assays. Protein assays used 10 fold diluted material. Rose Bengal assays used 0.30 mL of undiluted material. From the results (next chapter) I concluded that the 35-50% resuspendate had no FABP and that from 50 to 75% cut had the FABP.

DEAE Sephadex A-50 Chromatography

Each DEAE Sephadex A-50 column (2 x ~25 cm) was prewashed with 400 mL TSM and eluted with TSM in 80 drop fractions at ~15 drops per min. The pool from the Sephadex G-75 step was applied to this column. A gradient maker was used with 400 mL, each, of TSM and TSM- 0.35 M KCl. Fractions of 264 drops each were collected on a Pharmacia Redifrac fraction collector.

Chosen undiluted fractions were assayed at 280 nm and 0.30 mL samples from chosen fractions were assayed by the rose Bengal fraction assay. FABP containing

fractions were pooled and each pool volume was measured. Pools were reassayed by the rose Bengal pool assay, labeled, and frozen.

The percentage recovery for this step is calculated using the formula:

$$\% \text{ recovery} = \frac{\text{mg FABP recovered after A-50 chromatography} \times 100}{\text{mg FABP recovered after G-75 chromatography}}$$

Sephadex G-50 step

The pools from the DEAE Sephadex A-50 columns were each applied to a Sephadex G-50 column (2.5 x 70 cm). Each column was washed with 400 mL TSM and eluted with TSM at ~15 drops per min. Fractions of 80 drops were collected on Pharmacia Redifrac fraction collectors. Fractions were assayed at 280 nm and by the 560 nm, red charcoal method. FABP was pooled and pool volume was measured. Pools were reassayed for FABP, labeled and frozen. The 280 nm assay used chosen undiluted fractions. The 560 nm assay used 0.30 mL fractions.

The percentage recovery of FABP for this step is calculated using the formula:

$$\% \text{ recovery} = \frac{\text{mg FABP in G-50 pool} \times 100}{\text{mg FABP in A-50 pool}}$$

Biuret assay

Each type of sample was diluted so as to allow quantitative measurements with biuret reagent [59] Samples of the 50-70% AS cut were diluted 1:9 (samples were 0.30 and 0.60 mL). For G-75 pool dilution was 1: 4 (samples were 0.40 and 0.80 mL), The A-50 pools were diluted 1:1 (samples were 0.40 and 0.80 mL. A BSA standard (5 mg/mL) was included, undiluted (sample was 0.50 mL) with water. 2 mL of the biuret reagent

was next added to each test sample and allowed to stand for 20 minutes. Then the tubes were read at 540 nm.

Conductivity tests

For identification of the salt concentrations of fractions from DEAE-Sephadex columns, Tubes 5, 10, 14, 21, 30, 35, 40, 45 and 50 were used for conductivity measurement on a conductivity meter (Leeds-Northrop Co. Philadelphia, PA). Conductivities were converted to salt concentrations by use of a calibration curve and plotted against tube number.

Characterization of pork liver FABP in Sephadex G-50 pools

Molecular weight determination

Before proceeding, 0.4 mL of each FABP pool from Sephadex G-50 was diluted with TSM to 8 mL and assayed at 280 nm and 560 nm to check that its protein and FABP content were similar to those seen in the original pool. The FABP and protein content of each pool were found to be almost equal to the previous data and therefore usable. Marker proteins, 30 mL, of 1.5 mg/mL (e.g., 45 mg protein for 30 mL TSM) of ovalbumin, myoglobin and cytochrome-c were also prepared.

Then ~8 mL of each pool was loaded on a G-50 column (1.9 x 70 cm). Elution of 65 fractions of 75 drops each was carried out, and samples of chosen fractions were assayed at 280 nm and 560 nm. The unused pooled fractions were saved.

Next, ~8 mL samples of blue dextran and the 4 marker proteins (ovalbumin, cytochrome-C, myoglobin and chymotrypsin) were loaded onto the column one after the

other and eluted in the same way. The chromatogram fractions containing blue dextran were read at 620 nm. The chromatograms from the individual marker proteins were read at 280 nm to identify the marker positions in elutions. Blue Dextran was loaded more than once to check for a shift in V_e (V_e = elution volume). A graph was plotted with V_e/V_o (V_o = void volume) [59], for each marker protein on the x-axis and log of molecular weight on the y-axis. By extrapolating the V_e/V_o of FABP into the slope of the plot, its molecular weight was determined.

Comparison of ability of pork liver FABP to bind to rose Bengal and Oleate and Palmitate

Chromatography

The purified FABP pools from G-50 column were each concentrated to 4 mL and applied to a Sephadex G-50 column (1.95 x 70 cm) which was eluted at 8 drops/min. Ninety-drop fractions were collected. 280 nm readings were taken; both rose Bengal and [^3H] oleate/palmitate assays were performed. Then, the data were compared. This is a standard assay used in our lab and is described next. The following fatty acid binding assay solutions were prepared in plastic containers to prevent binding of the fatty acid to glass container walls:

Cold 11.6 mM Palmitate: 11.6mM palmitate stock in ETOH (32.7mg in 10mL).

Hot Oleate Stock: Titrated (^3H) fatty acid (New England Nuclear Corp, NEN), was purchased and stored in a -30°C freezer. Then: 40 μCi of this material was dissolved in 1.8 mL of ethanol with 0.2 mL of cold 11.6 mM oleate. This yielded 1.16 mM hot stock.

Hot Palmitate Stock: 1.16 mM ^3H -palmitate was prepared in the same way that the

oleate was prepared. It was also purchased from NEN.

50 mM Sodium phosphate: containing 150 mM NaCl (pH 7.0) was prepared by combining 100 mL of 0.5 M Na_2HPO_4 with 150 mL of 1 M NaCl in a 1-liter volumetric flask, neutralized to pH 7.4, stored in a refrigerator, and used for up to 6 weeks.

Preliminary G-50 Test

Exactly 0.5 mL of 1.16 mM ^3H -fatty acid stock was diluted with 4.5 mL of 50mM phosphate buffer. The diluted stock (16 μM) was made in a plastic test tube and discarded at day's end. To each plastic assay tube 1.00 mL of a mixture of 49.2 mL of phosphate buffer + 0.80 mL diluted stock (palmitate or oleate) 0.10 mL, chosen G-50 concentrate were added. This gave 1.93 mM oleate or palmitate in each reaction mixture. The tubes were mixed and allowed to stand 20 min at room temp. 0.10 mL charcoal-dextran was added, mixed, and allowed to stand for 15 min at room temperature. The tubes were then centrifuged for 10 min and 500 μL was taken to count.

Column Fraction Assay (after G-50 column in previous step)

This is done concurrently with 280 nm assay and pink assay for each of 4 experiments with each FA as described above. The samples were quickly counted for 1 min each. Then they were counted for 2 min. Oleate fractions were quick counted for 1 min, then recounted for 2 min. The process was repeated with palmitate.

Gel Electrophoresis

Gel electrophoresis was done in our Phast electrophoresis apparatus (Pharmacia-LKB, Piscataway, NJ) phast gel gradient 10-15% gels, as described in [62].

Its components were:

- 1) Stain, (0.1% phast gel blue solution in 30% methanol plus 10% acetic acid in DI water)

- 2) Destain (30% methanol and 10% acetic acid in D.I water)
- 3) Preserving solution (10% glycerol and 10% acetic acid in D.I water)
- 4) Denatured protein standards used were 1 mg/mL BSA and 1 mg/mL homogeneous rat FABP

The gel compartment was prepared for use by placing a gel in proper alignment and placing the phast gel buffer holder with 2 buffer strips onto the separation bed. The sample applicator was loaded with BSA, AMS Cut, G-75, A-50, G-50 and rat FABP in separate lanes. Prior to this, samples had been mixed with tracking dye and denatured by boiling for 5 minutes. Protein separation runs were performed and gels were allowed to dry and were scanned, to provide records, (see Results).

Electrofocusing

Column Isoelectric focusing (electrofocusing) was done as described by Pharmacia LKB [60]. Ampholine samples were made from a 2.5 mL aliquot of a 40% solution of ampholine, diluted with DI water to 10 mL of solution A. From this, a dense solution (7.5mL solution A + 42 mL DI water + 28g sucrose) and a less dense solution (2.5mL A + 60mL DI water) were prepared. Anode solution (0.2 mL of concentrated, H_3PO_4 + 14 mL DI water + 12g sucrose) and Cathode solution (0.2 mL ethylenediamine + 10mL DI water) were also prepared.

The electrofocusing column # 8100 was readied for each use by filling it with anode solution in the bottom by port no 1. Twenty four mixed ampholine solutions (Table 1) were then added to form the density gradient.

Table 1: Ampholine solutions containing different amounts of ampholines and sucrose (for density gradient)

| Tube # | Dense solution (mL) | Light solution (mL) | Tube # | Dense solution (mL) | Light solution(mL) |
|--------|---------------------|---------------------|--------|---------------------|--------------------|
| 1 | 4.6 | 0 | 13 | 2.2 | 2.4 |
| 2 | 4.4 | 0.2 | 14 | 2.0 | 2.6 |
| 2 | 4.2 | 0.4 | 15 | 1.8 | 2.8 |
| 4 | 4.0 | 0.6 | 16 | 1.6 | 3.0 |
| 5 | 3.8 | 0.8 | 17 | 1.4 | 3.2 |
| 6 | 3.6 | 1.0 | 18 | 1.2 | 3.4 |
| 7 | 3.4 | 1.2 | 19 | 1.0 | 3.6 |
| 8 | 3.2 | 1.4 | 20 | 0.8 | 3.8 |
| 9 | 3.0 | 1.6 | 21 | 0.6 | 4.0 |
| 10 | 2.8 | 1.8 | 22 | 0.4 | 4.2 |
| 11 | 2.6 | 2.0 | 23 | 0.2 | 4.4 |
| 12 | 2.4 | 2.2 | 24 | 0 | 4.6 |

Finally, the column was topped off to about 1 cm above the cathode with cathode solution. The FABP to be electro focused was added in several ampholine solutions in the middle of the density gradient (tubes 9-17). After the column is ready, electrodes are attached and electrophoresis is carried on at 100 volts (EPS 600, Pharmacia, Piscataway, NJ), overnight (~14 hours). Then the voltage is raised to 300 volts for 56-60 hours until amperage of 0.5 Amps is reached. The power is turned off, and 30 fractions (~3 mL) are collected in a Redifrac fraction collector (Pharmacia LKB, Piscataway, NJ). Finally, pH readings are taken for the fractions and they are assayed for FABP content.

CHAPTER IV

RESULTS AND DISCUSSION

Clofibrate and fenofibrate studies in SHR and Holtzman Rats

Our efforts here were carried out on 2 and 20 month male spontaneously hypertensive rats (SHRs) and male Holtzman normotensive rats (HoRs). I explored fatty acid levels in male SHRs, and HoRs plus or minus fibrates. This study was done in collaboration with Matthew Boka [67] to study the effects of drugs used in treating cardiovascular disease on the FABP levels in rat liver and intestine as well as the LFABP/IFABP ratio. We focused on clofibrate and fenofibrate, to show their relation to lipid-level alteration and to identify whether drops in blood pressures and elevated LFABP/IFABP ratios were similar to data we took with younger SHRs.

We compared levels of FABPs, LFABP/IFABP, and blood pressure, in SHR controls and SHRs given the test drugs clofibrate and fenofibrate. This allowed us to extend data already obtained by others in the group with clofibrate and to compare the differences in the basis for action of the 2 fibrates. I compared reported anti-cardiovascular potency of clofibrate with fenofibrate potency by examining LFABP/IFABP ratios in male rats given the drugs.

Results of SHR clofibrate study

[LFABP]/g tissue, shown in Table 2 and per 100 g body weight (not shown) increased significantly after clofibrate diet. IFABP did not increase significantly. A significant increase in LFABP/IFABP per 100g body weight also occurred in the animals

given clofibrate. The blood pressures in the treated SHR rats decreased significantly by 24 mm Hg, as expected.

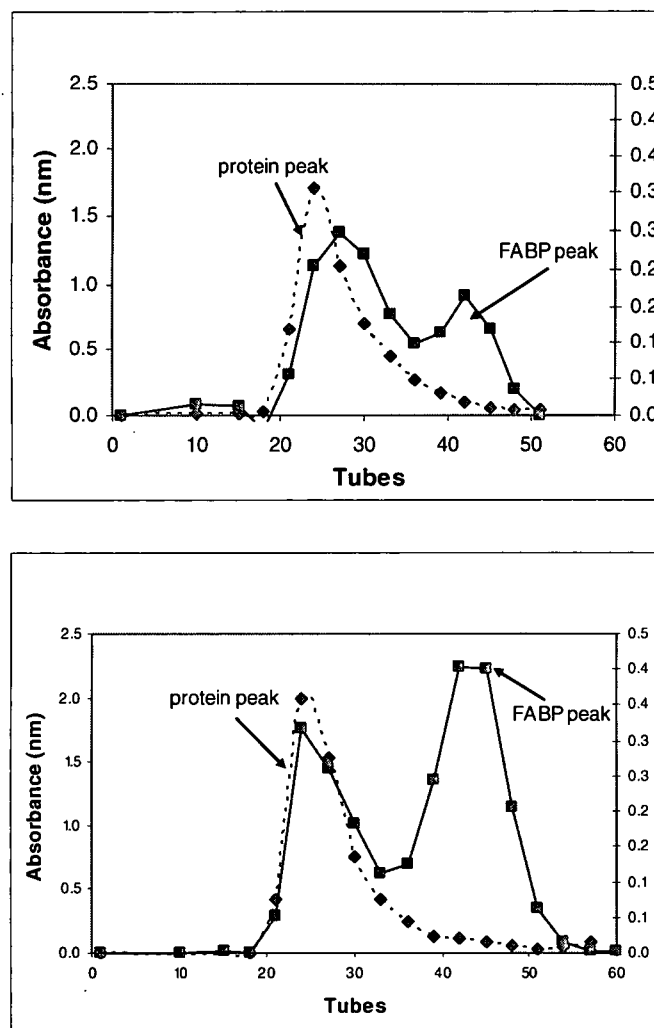


Figure 1 (top) & Figure 2 (bottom): Typical chromatograms that represent the significant increase in LFABP in SHR rats given clofibrate diet (right) Vs SHR rats given control diet (left). The chromatograms represent 1 of 8 experiments, each carried out on experimental and control rats.

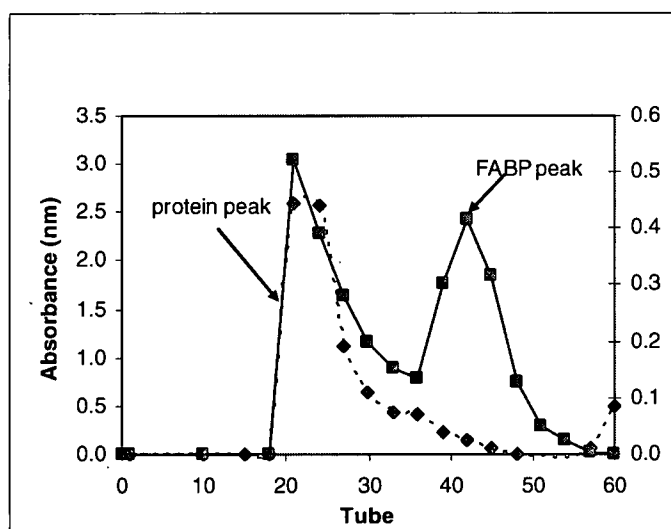
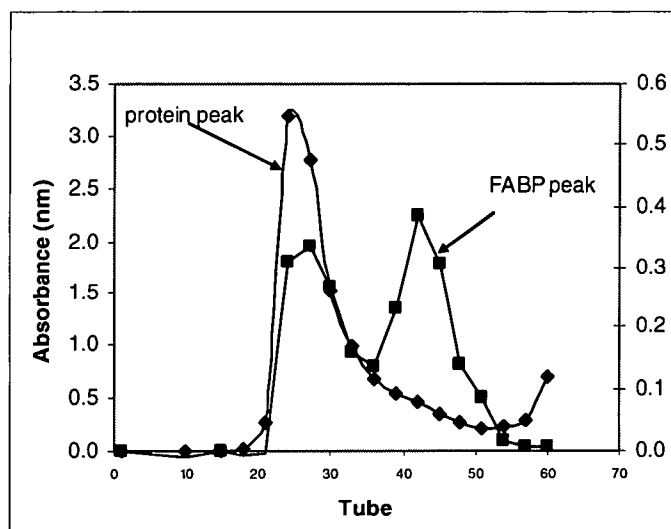


Figure 3 (top) & Figure 4 (bottom): Typical chromatograms that represent the slight increase in ILFABP in SHR rats given clofibrate diet (right) Vs SHR rats given control diet (left). The chromatograms represent 1 of 8 experiments, each, carried out on experimental and control rats.

Results from Hetman rat and SHR fenofibrate oral intubation

Here, the LFABP per g liver and per 100 g bodyweight increased significantly in Holtzman rats given fenofibrate. While [IFABP] per g mucosa and per 100 g body weight also increased to a smaller extent. The LFABP/IFABP per g tissue and per 100 g body weight also increased in the rats given fenofibrate. This set the stage for study of the

effect of fenofibrate in SHR by showing a significant increase in the LFABP/IFABP, in response to a second fibrate drug and that blood pressure decreased significantly by $26 \pm \text{SEM}$ mm Hg. The overall data are presented in Table 2.

Table 2: Effects of cardiovascular drugs on LFABP, IFABP, LFABP/IFABP of SHR and Holtzman rats

| EFFECTS OF CARDIOVASCULAR DRUGS ON LFABP, IFABP, LFABP/IFABP *Number of animals per Group *Statistically significant difference from controls $P < 0.05$ | | | | | | |
|--|----------------------------|-----------------|------------------------------------|-----------------|-------------------|-------------------|
| Measured quantity/g | Study with clofibrate diet | | Studies with intubated fenofibrate | | | |
| | SHR clofibrate | | HOLTZ fenofibrate | | SHR fenofibrate | |
| | EXPT (8)* | CONT (8)* | EXPT (9)* | CONT (7)* | EXPT (6)* | CONT (6)* |
| LFABP | $2.13 \pm 0.12^+$ | 1.12 ± 0.11 | $2.68 \pm 0.14^+$ | 1.03 ± 0.07 | 2.42 ± 0.10 | $0.89 \pm 0.47^+$ |
| IFABP | 0.77 ± 0.12 | 0.55 ± 0.07 | 1.12 ± 0.12 | 0.43 ± 0.02 | 0.95 ± 0.08 | $0.51 \pm 0.04^+$ |
| LFABP/IFABP | $86 \pm 0.89^+$ | 2.21 ± 1.48 | 2.64 ± 2.04 | 2.43 ± 0.38 | $2.63 \pm 0.29^+$ | 1.80 ± 0.85 |

SHR clofibrate per g tissue, shown above and per 100 g body weight (not shown) increased significantly after clofibrate diet. IFABP did not increase significantly. A significant increase in LFABP/IFABP per 100 g bodyweight also occurred in the animals given clofibrate. The blood pressures in the treated rats decreased significantly by $24 \pm \text{SEM}$ mm Hg ($P < 0.05$).

Holtzman and SHR fenofibrate oral intubation study: Here the LFABP per g liver and per 100 g body weight increased significantly in Holtzman rats given fenofibrate. While [IFABP] per g mucosa and per 100 g bodyweight increased to a smaller extent. The LFABP/IFABP per g tissue and per 100 g bodyweight also increased in the rats given fenofibrate. This set the stage for study of the effect of fenofibrate on SHRs by showing a large increase in the LFABP/IFABP and proving that rats survived the drug

dose. So, I next examined the fenofibrate effect in SHR rats. The data showed that LFABP/IFABP was altered significantly, in response to a second fibrate drug and that blood pressure decreased significantly by $25 \pm \text{SEM}$ mm Hg.

Purification of LFABP from pork liver

As indicated in the Materials and Methods section, homogenates from different pork liver samples were subjected to ammonium sulfate (AS) cuts (35-50%, and 50-75%). The 35-50% AS cut precipitated undesired tissue proteins present in the homogenate. Upon centrifugation, the FABP was in the supernatant. After the 50-75% AS cut the supernatant did not contain FABP and hence was discarded. TSM buffer was then used to dissolve the precipitate which contains the FABP.

In one of the six preparations carried out, the homogenate volume was 224 mL. The dissolved pellet from the 50 to 75% cut was 40 mL. The dissolved pellet from the 50 to 75% cut was loaded onto a G-75 column.

Sephadex G-75 chromatography

For the first set of experiments 100 g pork liver samples were used. The samples were homogenized in TSM; homogenates were centrifuged to obtain supernatants which were then subjected to consecutive AMS cuts {0-35%, 35-50%, 50-75%}. After centrifugation each pellet was resuspended in enough TSM to bring the volume of the resultant solution to 12-15 mL. The resuspended 35-50% and 50-75% cut material were each chromatographed on a Sephadex G-75 column {1.4 cm x 70 cm}. After chromatography, fractions were chosen for protein {280nm} and pink {560 nm} assays. Protein assays used 10 fold diluted material. FABP assays used 0.30 mL of undiluted material. The maximum yield of FABP was obtained in the samples from the 50-75% cut. It made up $8.1 \pm 1\%$ of the FABP present in the tissue

The tissue samples were then scaled up from 100 g to 200 g on a Sephadex G-75 column {1.9 cm x 70 cm}. After chromatography, 280 nm and pink assays to locate FABP were carried out, FABP was pooled, and then the pools were assayed to quantify LFABP. Protein assays used 10 fold diluted material. Rose Bengal assays used 0.30 mL of undiluted material. Fig. 7 is a sample chromatogram. A total of 12 experiments were done.

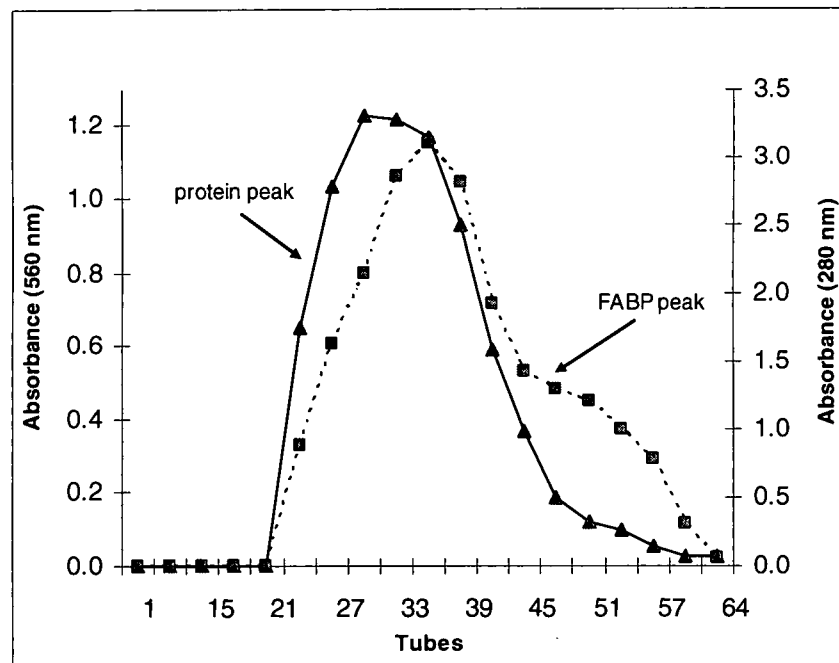


Fig 5: Protein and rose Bengal Assay plot of a Sephadex G-75 pool from the 50 to 75% cut of pork liver homogenate

The protein peak shows 280 nm readings which represent proteins other than FABP. The 2 prominent rose Bengal FABP peaks show 560 nm readings, of which the smaller and wider peak indicates where FABP is located in the Sephadex G-75 fractions. In the chromatogram shown, fractions 44 to 62 contained FABP. The pool volume was 80 mL of which 7 mL was saved and 73 mL was loaded onto a Sephadex G-50 column. The mg FABP/g [Total RBU/ (3.69 x corrected weight)] obtained was $\sim 15.5 \pm 1.4\%$ of what we saw in male rats.

Table 3: Representing the data obtained from assaying Sephadex G-75 pool

| | | | | |
|--|--|--|--|-------|
| Calculations: | | | | |
| 0.67 x RBU/mL | | | | 1.6 |
| Total RBU (0.67 RBU/mL x pool volume) | | | | 129.6 |
| mg FABP/g [Total RBU/(3.69 x corrected weight)] | | | | 0.17 |

$$\% \text{ recovery} = 0.17 / 0.9 * 100 = 19 \%$$

DEAE Sephadex A-50 step

Each Sephadex G-75 pool was applied to a DEAE Sephadex A-50 column (2 x 24-26 cm, 400 mL TSM prewash) and eluted with TSM in 80 drop fractions at ~15 drops per min. Fractions were assayed at 280 nm and at 560 nm. FABP was pooled and the pool volume was measured. Pools were reassayed with red charcoal, labeled, and frozen. Figure 6 shows a typical chromatogram.

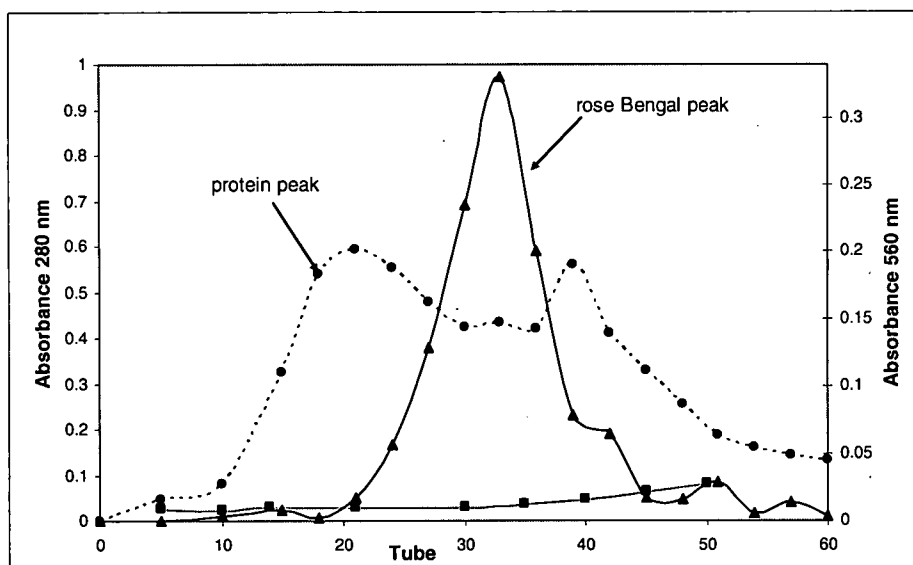


Fig 6: Typical chromatogram showing protein and pink assay plot of DEAE Sephadex A-50 chromatography.

A comparison between Sephadex G-75 and DEAE Sephadex A-50 steps was made to determine the effectiveness of FABP recovery between the 2 steps. This showed how much FABP was recovered between each step and the percent yield as shown in the above table.

Sephadex G-50 step

The DEAE Sephadex A-50 pool was loaded onto a Sephadex G-50 column, which was pre-washed with TSM. The fractions collected were analyzed at 280 nm and 560 nm.

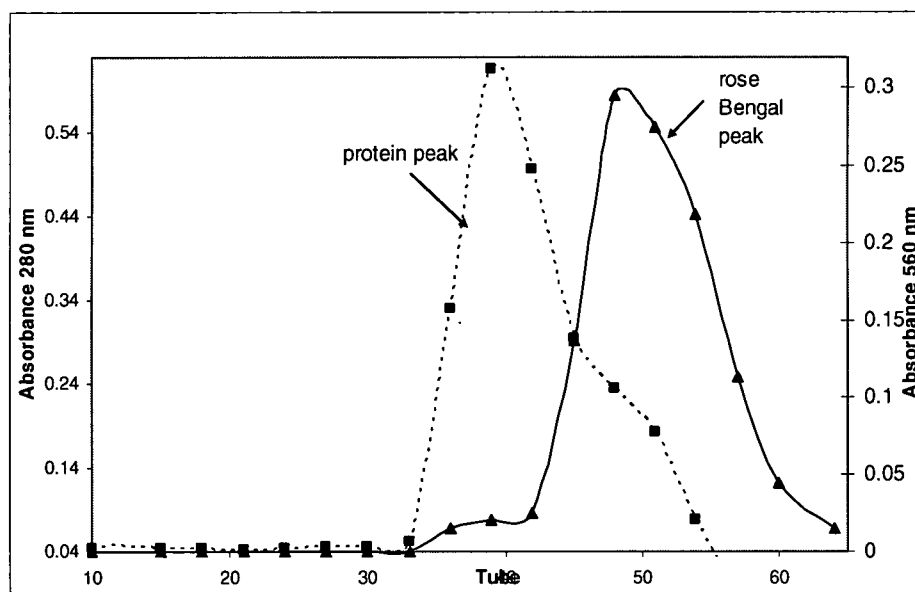


Figure 7: Typical chromatogram showing protein and pink assay plot of Sephadex G-50 chromatography. This was one of 5 similar experiments

As seen in Figure 7 the absorbances were graphed against the fraction numbers. There was one peak which contained FABP. The fractions pooled in this experiment are numbers 48 to 64 and the pool volume was 66.4 mL. The pool was analyzed for FABP at 560 nm. Comparison was made between A-50 and G-50 pools to determine the FABP recovery between the 2 steps. It made up 87 ± 8 % of the FABP present in the tissue.

Table 5: Representing the data obtained from assaying Sephadex G-50 and DEAE Sephadex A-50 pools

| DEAE Sephadex A-50 pool | | | | Sephadex G-50 pool | | | |
|--------------------------|--------|------|------|---------------------------|--------|------|------|
| Tube | mLFABP | A560 | A/mL | Tube | mLFABP | A560 | A/mL |
| 1 | 0.05 | 0.10 | 2.00 | 1 | 0.05 | 0.00 | 0.00 |
| 2 | 0.1 | 0.21 | 2.11 | 2 | 0.1 | 0.14 | 1.40 |
| 3 | 0.2 | 0.39 | 1.97 | 3 | 0.2 | 0.21 | 1.05 |
| 4 | 0.3 | 0.52 | 1.73 | 4 | 0.3 | 0.25 | 0.84 |
| 5 | 0.4 | 0.63 | 1.59 | 5 | 0.4 | 0.27 | 0.69 |
| Average activity/ ml= 62 | | | | Average activity/ ml = 59 | | | |
| Yield = 96% | | | | | | | |

The amount of FABP recovered between each step was significant and improved with each step. This is evidenced by the significant % recovery in each step.

Table 6: Representing the calculation of total fold purification between the Sephadex G-75 (G-75) and DEAE Sephadex A-50 (A-50) steps

| Quantity Indicated | AS Cut | G-75 | A-50 |
|--------------------------------|--------|-------|-------|
| Average pink absorbance | 104.4 | 187.8 | 133.7 |
| Average biuret absorbance | 3090 | 705 | 415 |
| Specific activity | 0.034 | 0.266 | 0.322 |
| Fold purification in each step | | 7.8 | 1.2 |

Table 7: Representing the calculation of total fold purification between A-50 and G-50 steps

| Quantity Indicated | A-50 | G-50 |
|---------------------------|------|------|
| Average pink absorbance | 62 | 59.4 |
| Average biuret absorbance | 17 | 13 |
| Specific activity | 3.64 | 4.24 |
| Fold purification | | 1.2 |

Total Purification

A progressive increase in % recovery of FABP supports the effectiveness of the successive chromatography method. The results of the Biuret assay of pools were compared with the pink assay results to show the actual purification of FABP compared with total protein content (Table 6), as denoted by increased specific activity (SA). There is an assumed correction factor of 24 fold from homogenate to the AS cut that could not be measured due to the intense red color of the solutions up to this point. However it was shown in other FABP preparations carried out in our lab [58]. Total purification: $24 \times 7.8 \times 1.2 \times 1.2 = 270$ fold.

The specific activity increased between each step. The fold purification (purification between each step) was determined by dividing the SA of the particular step by the SA of the previous step [e.g.: (SA G-75/ SA as cut = $0.266/0.0340 = 7.8$). The total purification in this preparation was found to be 270 fold. The mean total purification was 211.8 ± 60 fold.

CHARACTERIZATION OF PURIFIED FABP

After purification by the above mentioned procedures the FABP I isolated was tested for purity and characteristics such as its molecular weight, binding characters etc by various techniques whose results which will be described in this section. These include determination of its molecular weight, gel electrophoresis, fatty acid binding studies, and isoelectric focusing. The studies also enabled the comparison of differences between the FABP isolated from pork liver and various other sources.

Determination of the Molecular weight of the FABP

The molecular weight of the FABP preparation was determined by Sephadex G-50 chromatography of 10 mL of the pool from the Sephadex G-50 step (page 31). The same volumes of blue dextran, ovalbumin, cytochrome-C, myoglobin and chymotrypsin were loaded onto the column one after the other and 65 fractions of 75 drops were collected in each case. The fractions of blue dextran were read at 620 nm and the rest of the protein fractions were read at 280 nm. Figure 8 shows the data collected for one of 6 similar experiments.

Table 8: Determination of the molecular weight of the FABP

The V_e of the blue dextran is the V_o for the experiment. It is 30.

| PROTEIN | V_e | MW | V_e/V_o | log MW |
|-------------------|-------|------|-----------|--------|
| Ovalbumin (OV) | 33 | 40 | 1.1 | 1.6 |
| Myoglobin (MY) | 41 | 17.5 | 1.4 | 1.2 |
| Cytochrome-C (CY) | 46 | 12 | 1.5 | 1.1 |
| Chymotrypsin (CH) | 37 | 25 | 1.2 | 1.4 |
| FABP | 48 | 12.6 | 1.5 | 1.1 |

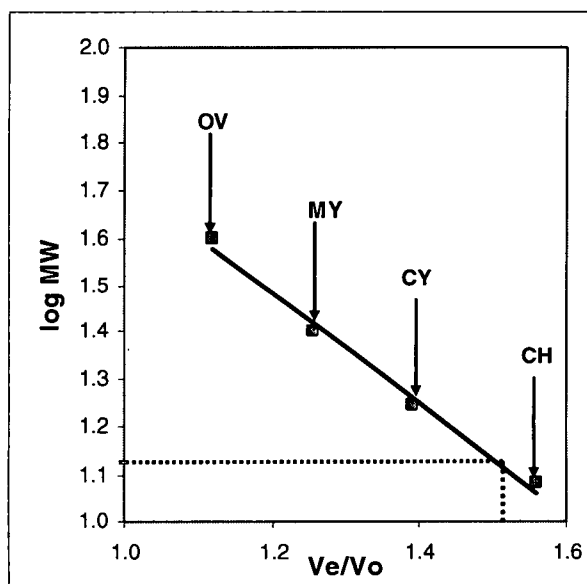


Figure 8: Plot of V_e/V_o vs $\log MW$ (determination of molecular weight of FABP).

From the above plot, the molecular weight of the FABP in the experiment shown was found to be 11, 200 Da (11.2 kDa). The mean molecular weight of the FABP was 15.6 ± 4.5 kDa averaged from 4 experiments.

Comparison of the Ability of Pork liver FABP to bind to rose Bengal,

Oleate and Palmitate

The purified FABP pools from pork liver were concentrated to ~4 mL and applied to a Sephadex G-50 column (1.95x70cm), which was eluted at 8 drops/min. Ninety drop fractions were collected, 280 nm readings were taken, rose Bengal & 3H -oleate/palmitate assays were performed, and the data were compared. Figure 9 shows one of three experiments which gave similar results.

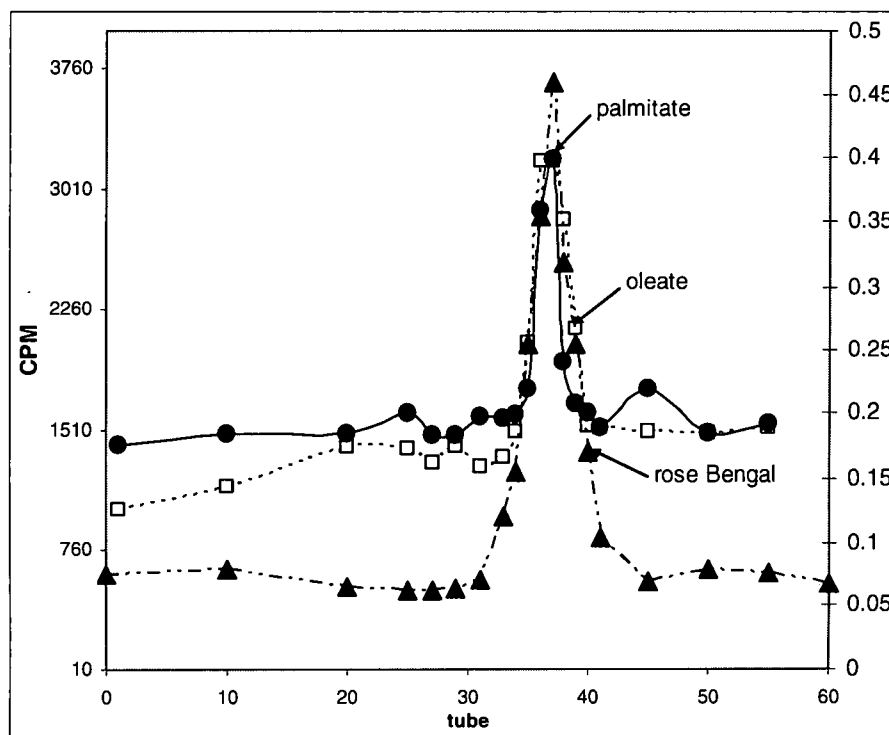


Figure 9: Plot representing radioactive studies using oleate, palmitate and FABP

The radioactive binding studies clearly indicate that FABP binds to oleate more than palmitate. This was one of 4 similar experiments.

Gel electrophoresis

The gel electrophoresis was used to compare the purity of the sample FABP to purified 98% pure BSA. The BSA; AMS Cut, G-75, A-50, G-50 (from pork liver); and rat FABP in lanes 1-6, respectively. The result was that a single FABP was purified. The gel electrophoretogram shows (Figure 10) that the pork liver FABP obtained from the final step is more distinct, and thus more highly purified, than that obtained from the earlier ones.

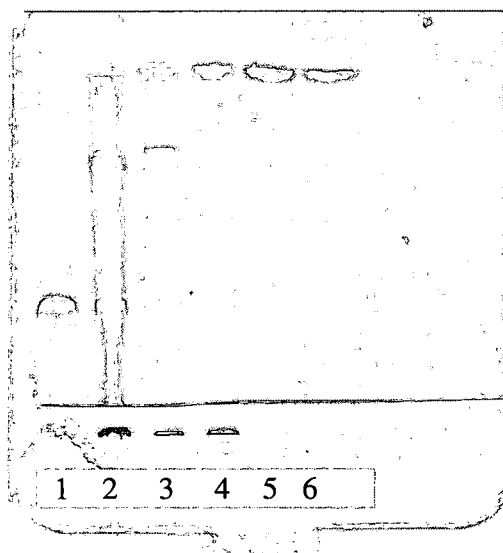


Figure 10: Sample gel electrophoresis.
This was one of 3 similar experiments

Isoelectric focusing

A rose Bengal assay was performed on the IEF fractions. The 560 nm absorbance data and pH data were graphed versus the fraction number (Figure 11). The rose Bengal assay results show two different 560 nm peaks which represent two types of FABP isolated.

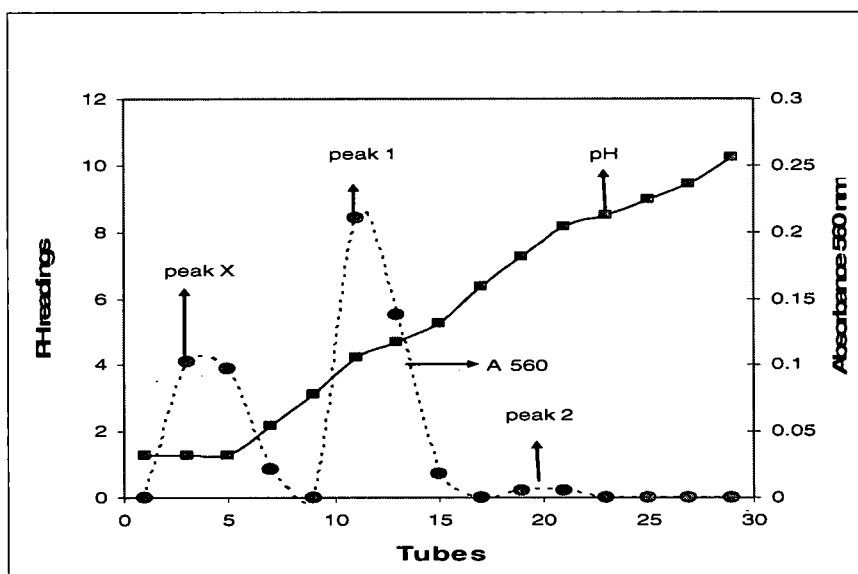


Figure 11: Isoelectric Focusing-Appearance of 2 peaks. One of 3 experiments.

The pH readings increased gradually with the tubes on the electrofocusing plot. Rose Bengal assay was performed on the IEF fractions. The 560 nm absorbance data and pH data were graphed versus the fraction number (Figure 11). The rose Bengal assay results show two different A-560 peaks which represent two types of FABP isolated. The main FABP peak (Peak 1) was at pH 4.2. Peak 2 at pH 8.1 is a minor FABP peak. Peak X at pH 1.3 was colorless and probably represents refractance. A significant amount of FABP was purified and isolated. However, the FABP sample was not homogenous since two types of FABP were present. The IEF data are similar to study results by others (chicken liver FABP by Ravichand Yalamanchili) [66].

CHAPTER V

CONCLUSIONS

We achieved the purpose of our experiments, the objectives of which were to study the effects of cardiovascular drugs on the levels of rat FABP and to develop a method of isolation of FABP from supermarket pork liver, which is inexpensive, to characterize the pork liver FABP and to measure the difference in yield compared to FABP isolated from fresh rat tissue.

The planned endeavor will expand our ability to study FABP actions, by a model developed in our lab. This model began with our observation of great species differences of FABPs in liver and intestine of rats and beagles [58, 59]. Because beagles have cardiovascular problems and rats are relatively resistant to such disease, the model was explored in spontaneously hypertensive rats and rats given several hypertensive drugs.

Data taken thus far support the model in that: The LFABP/IFABP drops in spontaneously hypertensive rats, compared to normotensive controls. The anti-cardiovascular drugs clofibrate and fenofibrate raise the LFABP/IFABP. Giving clofibrate to SHR's raises LFABP/IFABP and decreases their blood pressures and reduces the CHD by increasing the HDL-cholesterol levels. Both fenofibrate and clofibrate studies concurred with the results of previous studies.

An adequately purified, inexpensive FABP preparation was made from the pork liver by using DEAE Sephadex A-50 and G-50 chromatography, which was suitable in quality and quantity for characterization of the FABP. The total purification in this preparation was found to be 270 fold. The mean total purification was 211.8 ± 60 fold.

The radioisotope and IEF results indicated that the sample of FABP was quite pure. The molecular weight of FABP was found to be 15.6 ± 4.5 kDa.

My data are also similar to data reported from fresh chicken liver [66]. Therefore, there are similar results between fresh rat tissue and frozen pork tissue FABP isolation.

CHAPTER VI

FUTURE DIRECTIONS

The current rat studies gave conclusive evidence of decrease in blood pressure due to changes in LFABP and IFABP levels. Further quantitative studies can be carried out to correlate the effects of fibrates on HDL and LDL cholesterol levels and changes in body weight. In addition, the effects of other cardiac drugs can be studied on the FABP levels in various tissues and also the FABP studies can be carried out to the next level, i.e. monkeys and human beings which may provide a significant breakthrough in cardiac research.

A homogenous sample of FABP was not obtained in this work. Potential experimentation that can be performed is the further purification. For example, the IEF can be scaled up so as to further separate peaks 1 and 2. Successive chromatography is another option to enhance purification. Also, other chromatography procedures can be performed, such as using a Sephadex G-75 chromatography column. In addition, the FABP can be characterized further. The two IEF peaks can be explored and proven to be FABPs.

Ligand-binding and immunoblot assays can also be performed. Further characterization can be carried out by cNMR and HPLC studies. One study includes exploration of the tie between blood fatty acid content/levels and drug effects on LFABPs, by using HPLC studies.

CHAPTER VII

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