PHYSIOLOGICAL MECHANISMS OF NUTRIENT TRANSPORT: CALCIFEROLS AND VITAMIN D-BINDING PROTEIN

by

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B.Sc. University of Victoria 2007

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE
In the
Department of Biomedical Physiology and Kinesiology

SIMON FRASER UNIVERSITY
Spring 2011

APPROVAL

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Degree: **Master of Science**
Title of Thesis: **Physiological Mechanisms of Nutrient Transport: Calciferols and Vitamin D-Binding Protein**

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Date Defended/Approved: **April 19, 2011**

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ABSTRACT

Biologically active metabolites of vitamin D affect cell growth and differentiation, and thereby contribute to physiological regulation. Vitamin D-binding protein (DBP) binds 25-hydroxycholecalciferol and other D metabolites, and participates in their delivery to cells. Based on evidence for receptor-mediated endocytosis (RME) of DBP, experiments were performed to analyze DBP transport in animal tissues and cells. The results provide evidence that DBP RME can occur through a pathway that differs from the well-characterized clathrin-dependent endocytic pathway of transferrin (Tf, circulatory iron carrier protein). Moreover, cell growth density has differential effects on (a) the endocytosis of Tf and DBP, and (b) epidermal growth factor-mediated stimulation of DBP endocytosis. Comparative analyses of tissues and cells provides evidence for possible hormonal (e.g., estradiol) and aging effects on transport and receptor-binding parameters of DBP and other nutrient carrier proteins.

Keywords: calciferols, endocytosis, metabolism and nutrition, nutrient transport, vitamin D-binding protein

Subject Terms: biochemistry, cell biology, metabolism, nutrition

EXECUTIVE SUMMARY

The calciferols collectively make up the different forms of vitamin D, and include the two most physiologically relevant ones, ergocalciferol (D2) and cholecalciferol (D3). Vitamin D3 is the precursor of biologically active, hydroxylated calciferols such as calcidiol (25(OH)D3) and calcitriol ($1,25(OH)_2D3$), both of which affect cell growth, differentiation, and gene expression through interaction with nuclear vitamin D receptors (VDR). Active metabolites have roles in maintaining bone structure and growth, providing neuroprotection, suppressing carcinogenesis, and protecting against autoimmune diseases such as multiple sclerosis. Vitamin D can be obtained from dietary sources, or from direct production in the body through a process that requires ultraviolet B radiation. Vitamin D and its biologically active metabolites are transported throughout the body via the vitamin D binding protein (DBP). Overall, the role of DBP in vitamin D physiology remains poorly understood. A major objective of this thesis was to gain a more systematic and detailed understanding of DBP transport and physiology. In order to obtain novel information, experiments were completed with the following specific goals:

- to characterize DBP endocytosis in cells, with respect to its occurrence and the type of endocytic pathway;
- to determine the effects of potential modulators such as growth factors (epidermal growth factor, EGF) on the rate of DBP endocytic transport
- to compare accumulation of injected, labelled-DBP in tissues of male

and female mice;

- to compare DBP binding capacities on crude tissue membrane fractions of male/female, and young/old, mice;
- to compare the above DBP transport-related parameters with those of other nutrient carrier proteins such as transferrin (Tf) and retinol binding protein (RBP).

The results of the experiments provide evidence for the existence of functional DBP receptors on the surface of human A431 cells, and in murine cells of the liver, lung, kidney, adipose, and brain. In comparison with transferrin, a well-characterized standard for cell transport studies, a higher level of DBP-binding activity is found on A431 cells, both on intact cells and isolated cell membrane fractions. Studies with endocytic inhibitors show that filipin significantly decreases the amount of DBP endocytosed in hepatocytes, a result consistent with a caveolar endocytic pathway for DBP. Evidence is also obtained that endocytic transport of DBP and Tf may be influenced by cell growth status. When comparing endocytic efficiency, it is found that at low cell-growth densities, Tf endocytosis is significantly more efficient than that of DBP. Moreover, the presence of EGF appears to stimulate DBP endocytic transport, but not at high (≥90 %) cell growth densities. Total DBP membrane binding capacity, however, is similar regardless of EGF treatment or cell growth density. Studies with the redox-sensitive indicator, MTT, provide evidence for a small, but significant increase in cell metabolic activity with EGF treatment at high cell growth densities. Taken together, the EGF results suggest that an

increase in metabolic activity—which could potentially give rise to an increase in cell proliferation—is inversely correlated with DBP endocytic efficiency. This is in agreement with the potential pro-differentiative, anti-proliferative effects of vitamin D.

Gender differences exist with regard to DBP endocytosis, and these differences correlate with the effect of sex hormones on DBP endocytic transport in isolated cells. Overall, *in vivo* (liver tissue) and *in vitro* (isolated hepatocytes) experiments both suggest that male liver cells have a decreased DBP endocytic efficiency relative to female liver cells. Moreover, isolated female hepatocytes in the presence of estradiol exhibit a significantly greater increase in DBP endocytosis relative both to non-estradiol-treated female hepatocytes and to male hepatocytes.

When comparing total binding activity of crude membrane preparations in different mouse tissues, liver is found to have a higher DBP binding activity than the others: lung, kidney, adipose and brain. The contribution of non-specific binding to these differences, however, is not known. When comparing binding to similar membrane extracts prepared from liver, lung, and kidney of old and young mice, statistically significant differences in total binding activity are observed between old and young liver and old and young kidney; in both cases, there is a decrease in the total binding activity in the older tissues. In conclusion, this thesis provides novel findings regarding the physiology of DBP transport, as well as novel comparisons of these findings with other nutrient carriers such as Tf and RBP.

DEDICATION

I would like to dedicate this work to my loving parents and to my dear fiancée, for their constant support, encouragement and love.

ACKNOWLEDGEMENTS

There are many people whom I would like to thank for contributing to the achievement of my Master's degree.

Firstly, I would like to thank my senior supervisor, Dr. Amandio Vieira, for allowing me the privilege to work with him. His unwavering support and guidance were key to my timely success and I can only hope that other graduate students can be as lucky as I to have such an invaluable mentor.

I would like to thank Dr. Allan Davison for his ever-insightful and detailed suggestions and comments throughout my thesis project. I am grateful for his eye-opening and knowledgeable advice in the areas of scientific writing and research. I am forever indebted to Vai Hong, whose patience, guidance and kind words were never-ending. I would also like to acknowledge Dr. Chris Kennedy for his time and constructive feedback with regard to my thesis.

I would like to recognize all my amazing professors who have graciously shared their knowledge and wisdom, Susie Nugent whose kindness, encouragement and help have been invaluable, and my close fellow grad-students who have provided friendship, understanding and balance.

Last, but nowhere near least, I would like to thank my loving parents and my dearest fiancée for their incessant encouragement, unconditional love and support; my family for their prayers and God, to whom all praise is due.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

b-X	Biotinylated-X (X = DBP, Tf, or other ligand)
BNHS	Biotin N-hydroxysuccinimide ester
BSA	Bovine serum albumin
CME	Clathrin-mediated endocytosis
CYP	Cytochrome protein
D2	Vitamin D2 or ergocalciferol
D3	Vitamin D3 or cholecalciferol
DBP	Vitamin D binding protein
DMEM	Dulbecco's modified eagle medium
EB	Extraction buffer
EGF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Esa	Enzyme (E, peroxidase enzyme) complexed with streptavidin (Sa)
FBS	Fetal bovine serum
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRP	Horseradish peroxidise
KSHM	100mM Potassium acetate, 85mM sucrose, 20mM Hepes, 1mM magnesium acetate, pH 7.4

MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide
MW	Molecular weight
OPD	*Ortho*-phenylene diamine
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PI	Post-injection of animals or (p.i.) post-incubation of cells
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
RA	Retinoic acid
Raf	Rapidly accelerated fibrosarcoma (kinase)
RBP	Retinol-binding protein
RME	Receptor-mediated endocytosis
SDS	Sodium dodecyl sulphate
SFM	Serum free medium
SICM	Semi-intact cells and membranes
Src	Sarcoma Kinase
STAV-HRP	Streptavidin-HRP
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween 20

Tf	Transferrin
TTR	Transthyretin (original name: pre-albumin)
UVB	Ultraviolet B light
VDR	Vitamin D receptor (nuclear)

CHAPTER 1: INTRODUCTION AND MAIN OBJECTIVES

1.1 Overview of project and main objectives

Biologically active metabolites of vitamin D have a wide range of physiological functions based on their ability to affect cell growth and differentiation. Vitamin D is typically obtained from the diet, and can also be synthesized in the body upon exposure to ultraviolet B radiation. Vitamin D can be stored in the body, primarily in the liver, and transported bound to vitamin D-binding protein (DBP) through the blood to different body tissues. The molecular mechanisms by which DBP is internalized and vitamin D is absorbed by cells are incompletely understood.

The *main objectives* of this MSc thesis project were to obtain a more systematic and detailed understanding of (**a**) DBP endocytic transport, (**b**) the influence of cell growth and selected hormones on such transport, (**c**) potential gender differences on DBP tissue accumulation and cell transport, e.g., sex

hormones as potential modulators of transport, and **(d)** potential age effects on DBP tissue binding. Moreover, **(e)** comparative studies with other nutrient carrier proteins such as retinol-binding protein (RBP; carrier of vitamin A) and transferrin (Tf; carrier of iron) have also been included.

More specifically, experiments have been carried out with the following specific goals (each represents an attempt to obtain novel and important information in this field):

- to characterize DBP endocytosis in cells, with respect to its occurrence and the type of endocytic pathway;
- to determine the effects of potential modulators such as growth factors (epidermal growth factor) on the rate of DBP endocytic transport
- to compare accumulation of injected, labelled-DBP in tissues of male and female mice;
- to compare DBP binding capacities on crude tissue membrane fractions of male/female, and young/old, mice;
- to compare the above DBP transport-related parameters with those of other nutrient carrier proteins such as Tf and RBP.

The main experiments related to the physiology of DBP transport are shown schematically in Figure 1-1.

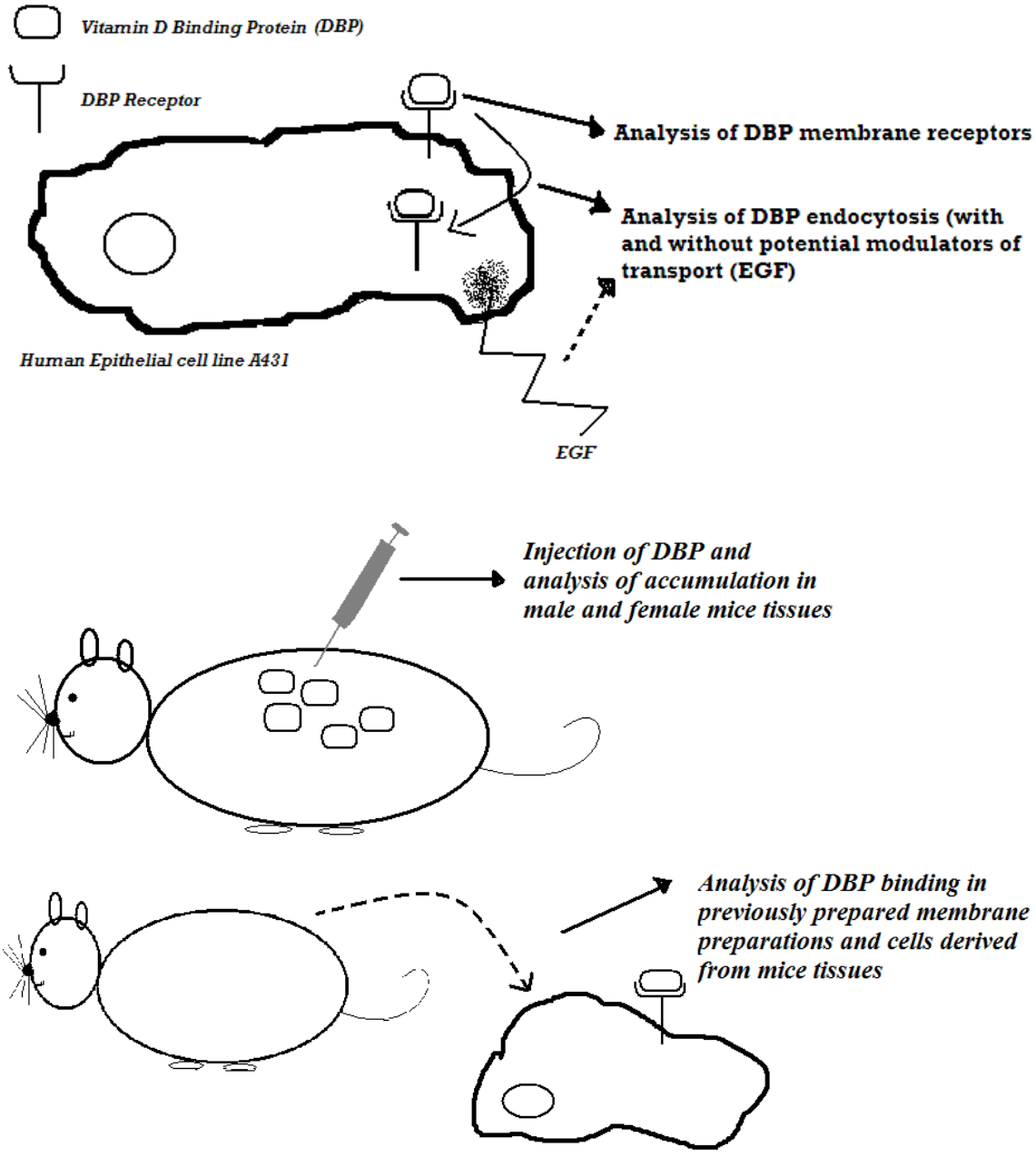

Figure 1-1 Summary of major experiments related to the physiology of DBP transport.

1.2 Background information

1.2.1 Vitamin D metabolites and their biological functions

Vitamin D forms are collectively known as the calciferols (or calcipherols). Five forms have been noted in the literature, including D2, ergocalciferol, D3, cholecalciferol, D4, 22,23-dihydroergocalciferol, D5, sitosterol, and D6, stigmasterol (Mehta & Mehta, 2002; Napoli, Fivizzani, Schnoes, & DeLuca, 1979); the historical term D1 is no longer used. The most physiologically important forms are D2 and D3. The D3 form is found in humans and other animals; in contrast, D2 is found in plants and typically forms less potent hormone derivatives in mammals.

Vitamin D3 is the precursor of biologically active, hydroxylated calciferols such as calcidiol (25(OH)D3, also called 25-hydroxycholecalciferol), the dominant circulating metabolite, 1-calcitriol ($1,25(OH)_2D3$, also called 1-alpha, 25-dihydroxycholecalciferol), the dominant active metabolite of vitamin D, and 24-calcitriol ($24,25(OH)_2D3$, also called 24,25-dihydroxycholecalciferol) (Rowling, Kemmis, Taffany, & Welsh, 2006; P. White & Cooke, 2000). Calciferol structures are shown in Figure 1-2.

The effects of biologically active calciferols have been studied in many physiological and pathological contexts, including those on gene expression, specifically regarding pro-differentiation, anti-proliferation, and apoptosis in cells (Colston, Colston, & Feldman, 1981), (Blutt, McDonnell, Polek, & Weigel, 2000). Vitamin D metabolites have been reported to increase innate immune system

defence mechanisms (Bhalla, Amento, & Krane, 1986; Holick, 2007; Ohta, Okabe, Ozawa, Urabe, & Takaku, 1985; van Etten & Mathieu, 2005) and to suppress carcinogenesis, particularly of colon, breast, ovarian and prostate cancers (C. F. Garland & Garland, 1980; C. F. Garland et al., 1989; F. C. Garland, Garland, Gorham, & Young, 1990; Lefkowitz & Garland, 1994; Schwartz & Hulka, 1990). Biosynthesis of 1-calcitriol by cells of the central nervous system has also been associated with neuroprotective effects due to up-regulation of neurotrophins (Garcion, Wion-Barbot, Montero-Menei, Berger, & Wion, 2002; Riaz, Malcangio, Miller, & Tomlinson, 1999; Y. Wang et al., 2000). Moreover, the positive effects of calciferols on bone structure, health and growth, and calcium and phosphate homeostasis, have been frequently reported (Bischoff-Ferrari, Dietrich, Orav, & Dawson-Hughes, 2004; Specker et al., 1992).

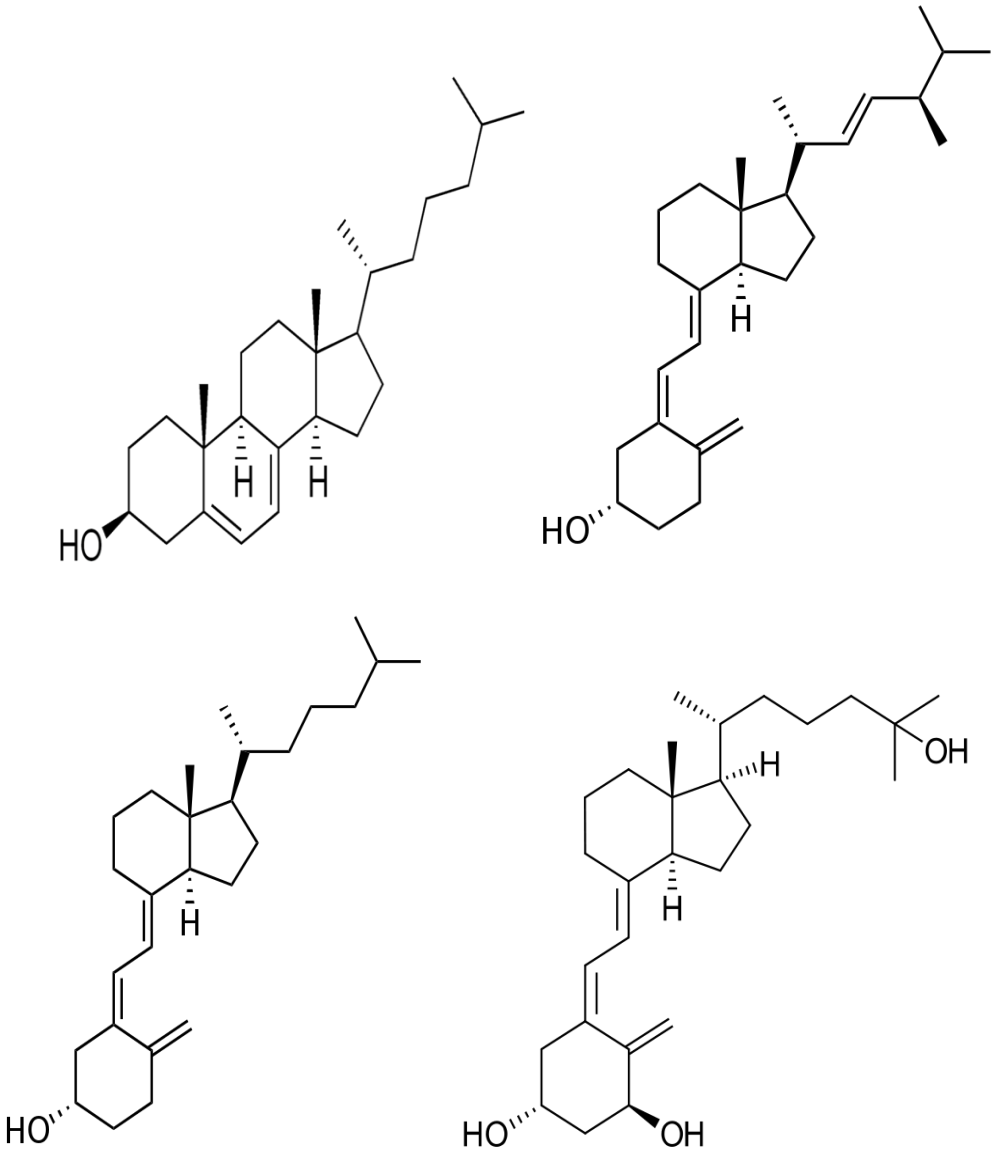

Figure 1-2 Chemical structures of 7-dehydrocholesterol and three calciferols.

7-dehydrocholesterol (pro-vitamin D) is the upper left structure. Vitamin D2, ergocalciferol, is shown in the upper right. And D3, cholecalciferol, in the lower left. The triple-hydroxyl calcitriol, 1,25(OH)$_2$D3, is shown in the lower right.

Dietary sources of vitamin D include fatty fish such as catfish, mackerel, salmon, sardines, and tuna, which provide the most abundant natural sources of Vitamin D, as well as liver, eggs or certain plants and fungi (Calvo, Whiting, & Barton, 2004; Koyyalamudi, Jeong, Song, Cho, & Pang, 2009). As well, many foods including milk and other dairy products, margarines, breads and cereals are fortified with Vitamin D, and vitamin D supplements are also available and commonly used (Calvo et al., 2004). Pro-hormone Vitamin D such as D3 is typically obtained from dietary sources; and after ingestion and absorption, it is transported via carriers such as chylomicrons or plasma binding proteins through the blood to various organs including the liver (Avioli, 1969; Dueland, Helgerud, Pedersen, Berg, & Drevon, 1983; Dueland, Pedersen, Helgerud, & Drevon, 1983).

Vitamin D3 can also be produced in the body from 7-dehydrocholesterol (7dC, also called pro-vitamin D) in a process that requires ultraviolet B light (UVB, 290-320 nm). In the body, 7dc is most concentrated in the stratus spinosum and stratus basale of the outer epidermis of the skin (Norman, 1998). In the presence of UVB, 7dC is converted to pre-vitamin D3, after B-ring photolysis and cleavage (See Figure 1-2), which then spontaneously isomerizes to the secosteroid D3 (Mehta & Mehta, 2002). D3 synthesis is dependent on skin pigmentation, but more importantly on substrate availability and quantity and quality of sun exposure; the amount of D3 synthesized is usually within about 15% of the amount of 7dC available (Norman, 1998; Webb, Kline, & Holick, 1988). Interestingly, studies have found that geographical latitude plays a role on the prevalence of diseases such as multiple sclerosis (van der Mei, Ponsonby,

Blizzard, & Dwyer, 2001), some cancers (Grant, 2003), inflammatory bowel disease (Sonnenberg, McCarty, & Jacobsen, 1991), osteoporosis (Hagenau et al., 2009), type 1 diabetes (Staples, Posonby, Lim, & McMichael, 2003) and rheumatoid arthritis (Cantorna, 2000); and there is evidence that vitamin D3 availability may decrease the risk of some of these diseases (Grant, 2003; Holick, 2003). It has been established that, although dietary sources provide immediate availability of D3, synthesis upon UV irradiation provides a more sustained and bioavailable source of this sterol (J. G. Haddad Jr & Hahn, 1973; J. G. Haddad, Matsuoka, Hollis, Hu, & Wortsman, 1993; Whyte, Haddad, Walters, & Stamp, 1979).

The vitamin D binding protein (DBP) carries D3 in the circulation to its activation sites in the liver, then to the kidney for further activation, and subsequently to many other tissues. DBP also transports the active forms of the vitamin such as calcidiol (P. White & Cooke, 2000). Overall, the role of DBP in vitamin D physiology remains poorly understood (see Section 1.2.2).

Synthesis of biologically active calciferols can occur in different tissues. Calcidiol is primarily synthesized in the liver and skin. Both calcitriol metabolites are synthesized predominantly in the kidney, where the enzymes 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1) and D-24-hydroxylase (CYP24) convert calcidiol to 1-calcitriol and 24-calcitriol, respectively (Schwartz, Whitlatch, Chen, Lokeshwar, & Holick, 1998; St-Arnaud et al., 2000; Zehnder et al., 2001). Found in the kidneys and the intestine, CYP24 is also able to convert 1-calcitriol to calcitroic acid, an inactive metabolite that is subsequently excreted in bile (Holick,

2008), or 24-calcitriol, the first substrate in the chain of events leading to the inactivation of vitamin D metabolites via the 24-oxidation pathway (St-Arnaud et al., 2000). In addition, active metabolite conversion can occur in immune cells such as active T cells (Sigmundsdottir et al., 2007), dendritic cells (Fritsche, Mondal, Ehrnsperger, Andreesen, & Kreutz, 2003) and macrophages (van Etten & Mathieu, 2005). As with most of the other fat-soluble vitamins, vitamin D is also stored in adipose tissue (Jones, Strugnell, & DeLuca, 1998).

Active forms of vitamin D can affect the expression of many genes by modulating their transcription. This is achieved through the interaction of these metabolites with the nuclear vitamin D receptor (VDR). Specifically, VDRs containing one of the active hydroxylated forms interact with a partner nuclear receptor protein (e.g., retinoid X receptor, RXR), with DNA, and with many other co-regulatory proteins (Garcion et al., 2002). For example, 1-calcitriol in neuronal cells has been shown to provide neuroprotection via several genetic regulatory mechanisms; by means of either up- or down-regulation of neurotrophin synthesis, this metabolite and its nuclear receptor, reduces hypokinesia and neurotoxicity (J. Y. Wang et al., 2001). Similarly, the effects of 1-calcitriol are accomplished through the up-regulation of γ-glutamyl transpeptidase and the reduction of both neuronal Ca^{2+} concentrations and inducible nitric oxide synthase (iNOS) production (Brewer et al., 2001; Dringen, Gutterer, & Hirrlinger, 2000; Garcion et al., 1998; Garcion, Sindji, Leblondel, Brachet, & Darcy, 1999). In the context of some cancers, the VDR 1-calcitriol-induced transcription factor is thought to increase expression of immune stimulating factors and the p75

receptor, lending to cell death, and decrease tenascin-C production resulting in reduced growth, invasion and angiogenesis (Alvarez-Dolado, Gonzalez-Sancho, Navarro-Yubero, Garcia-Fernandez, & Munoz, 1999; Furman, Baudet, & Brachet, 1996; Meldolesi, Sciorati, & Clementi, 2000). In animal models, treatment with 1–calcitriol has also been associated with the down-regulation of expression of various factors, including iNOS, and provides a protective effect against encephalitis and the progression of autoimmune diseases such as multiple sclerosis (Eikelenboom, Killestein, Kragt, Uitdehaag, & Polman, 2009; Garcion, Nataf, Berod, Darcy, & Brachet, 1997; Hayes, 2000; Lemire & Archer, 1991; Mattner et al., 2000).

In addition to biological effects of hydroxylated forms of vitamin D mediated by VDRs and changes in gene expression, there is evidence for more immediate biological effects that do not depend directly on changes in gene expression. Typically, these are effects at the plasma membrane and are much faster, taking seconds to minutes, when compared to genomic actions that can take several hours to days (Mizwicki & Norman, 2009). The initiation of intestinal calcium transport by 1-calcitriol was the first of these non-genomic responses to be clearly evidenced, after which several other responses have been confirmed. With binding to caveolae-associated VDRs, 1-calcitriol can initiate calcium signalling, modulate cell exocytic transport and promote PKC, PLC, PLA_2, MAPK, Raf, Src and G-protein activation through rapid non-genomic action (Bissonnette et al., 1994; Boyan et al., 1998; de Boland & Norman, 1998; Gniadecki, 1996; Gniadecki, 1998; Khare et al., 1994; Khare et al., 1997; Mizwicki & Norman, 2009;

Nguyen et al., 2004; Selles & Boland, 1991). Other responses mediated by 1-calcitriol include vascular smooth muscle cell differentiation, cell migration, sphingomyelin hydrolysis, insulin secretion, phospholipid metabolism and regulation of myocyte contraction and relaxation (Bhatia, Kirkland, & Meckling-Gill, 1995; Bourdeau, Atmani, Grosse, & Lieberherr, 1990; Gniadecki, 1996; Kajikawa et al., 1999; Rebsamen, Sun, Norman, & Liao, 2002; Tishkoff, Nibbelink, Holmberg, Dandu, & Simpson, 2008). Both the cell membrane and cell nucleus-associated receptors exhibit strong binding to vitamin D metabolites, with an affinity of approximately 1 nM (Norman, Olivera, Barreto Silva, & Bishop, 2002); however, nuclear-VDR exhibits stronger binding with 6-*s-trans* shaped 1-calcitriol ligand, while the caveolae-associated VDR exhibits stronger binding with 6-*s-cis* shaped ligand (Norman, Mizwicki, & Norman, 2004; Norman, 2008).

1.2.2 Vitamin D binding protein (DBP)

The 52 kDa DBP, mainly synthesized and secreted by hepatic cells in the liver, is present in human serum and has been detected in the sera of various animals including the rat and mouse, rabbit, turtle and chicken (Burnside & Sofer, 1999; Cooke & David, 1985; Cooke, 1986; Hunt & Licht, 1998; Osawa, Tsuji, Yukawa, Saito, & Takeichi, 1994; F. Yang et al., 1990). Serum concentrations in healthy individuals are usually found in the range of 300-600 mg/L (M. M. Speeckaert et al., 2008). DBP is a member of the albumin protein family; however, despite the close linkage of this gene family on human chromosome 4, the

functions of DBP differ greatly compared to those of its other family members including albumin, α-fetoprotein and α-albumin/afamin (P. White & Cooke, 2000).

Because DBP is present in the plasma at concentrations 20 times that of all its metabolites, the majority of vitamin D metabolites (>88% of calcidiol and >99% of calcitriol) in circulation are bound by this protein carrier under normal physiologic conditions (Bikle et al., 1986; M. Speeckaert, Huang, Delanghe, & Taes, 2006; P. White & Cooke, 2000). Typically, these DBP concentrations remain at consistent levels from infancy to adulthood, with the exception of greatly increased concentrations during pregnancy and with oestrogen therapy (M. Speeckaert et al., 2006; M. M. Speeckaert et al., 2010).

In addition to transport of sterols, DBP acts as a G-actin scavenger and, together with the serum protein gelsolin, helps to protect its host from actin-mediated damage in the microvasculature during events of chronic inflammation, trauma or necrosis (J. G. Haddad et al., 1992; Lee & Galbraith, 1992; P. White & Cooke, 2000). G-actin becomes bound to a C-terminus domain on DBP with high affinity (M. M. Speeckaert et al., 2010). Some studies have even shown that mortality caused by organ failures, particularly acute liver failure, can be predicted by determining actin-free DBP concentrations in the plasma (Lee et al., 1995; Schiodt et al., 1996; Schiodt et al., 2005); the lower the plasma levels of actin-free DBP the greater is the risk of organ failure. DBP may play a role in the transdifferentiation of hepatic stellate cells to myofibroblasts during fibrogenesis (Gressner, Lahme, & Gressner, 2008). In vitro studies have shown that after activation of DBP through de-glycoslylation by B- and T-cell enzymes, the

resulting DBP-macrophage-activating factor (DBP-MAF) complex, in turn, causes macrophage activation (Yamamoto, Homma, Haddad, & Kowalski, 1991; Yamamoto & Kumashiro, 1993; Yamamoto & Naraparaju, 1996). DBP has also been shown to bind to CD44 and annexin A2 binding sites on the cell surface of certain leukocytes, specifically macrophages and neutrophils, and increase their chemo-attraction to C5a, potentially recruiting these cells to sites of hepatic inflammation (Gressner et al., 2008; Kew & Webster, 1988; Perez, 1994; Piquette, Robinson-Hill, & Webster, 1994).

The crystal structure of human DBP is shown in Figure 1-3. It is composed of several alpha helices that create three domains: 10 helices make up the first domain, 10 also compose the second, and the third domain contains 4 helices (Verboven et al., 2002). Multiple allele variances have been identified for this polymorphic protein, three of which are most common and co-dominant (DBP 1-1, DBP 2-1 and DBP 2-2) (M. M. Speeckaert et al., 2010; P. White & Cooke, 2000). DBP and its other family members with homologous structures, albumin, α-fetoprotein, and afamin, all bind free fatty acids; but DBP differs slightly in its helical organization and this difference allows it to have unique functions such as binding of vitamin D (Ena, Esteban, Perez, Uriel, & Calvo, 1989; Williams, Van Alstyne, & Galbraith, 1988). Although it binds both vitamin D and various D analogs, DBP's affinity is highest for 1-calcitriol with a $K_a = 5 \times 10^8\ M^{-1}$ (P. White & Cooke, 2000). The binding site for vitamin D3 ligands is located in the N-terminal region of domain 1, from residues 35-49, helices 1-6 (Meier, Gressner, Lammert, & Gressner, 2006; Verboven et al., 2002).

The functions attributed to DBP also stem from its interaction sites for several cell-surface receptors including megalin. There is evidence that megalin is the primary receptor for DBP uptake in some tissues such as the kidney (Section 1.2.4.1). Other DBP receptors identified to date include cubilin, chondroitin sulfate proteoglycans, CD44/HCAM, and annexin A2 (DiMartino & Kew, 1999; Gressner et al., 2008; McVoy & Kew, 2005; Meier et al., 2006; Nykjaer et al., 2001).

DBP can interact with various lipoproteins. In addition to its significant correlation with total plasma cholesterol ($r=0.40$; $p<0.0001$), serum DBP concentrations were also highly correlated to both LDL-cholesterol ($r=0.34$; $p<0.005$) and triglyceride ($r=0.65$; $p<0.0001$) levels (M. M. Speeckaert et al., 2008). Studies using various precipitation methods have found that DBP co-precipitates in part with VLDL and LDL; this result indicates that a fraction of circulatory DBP may be bound to these lipoproteins (M. M. Speeckaert et al., 2008; M. M. Speeckaert et al., 2010, M. Speeckaert et al., 2006). Because of its strong lipoprotein associations, DBP may be a potential nutritional status marker, particularly with regard to lipids. This is especially significant for cystic fibrosis patients—whose mean DBP serum concentrations are known to be considerably (18-20%) lower than healthy individuals—as body weight and body fat are determining factors in their survival (Coppenhaver et al., 1981; Corey, McLaughlin, Williams, & Levison, 1988).

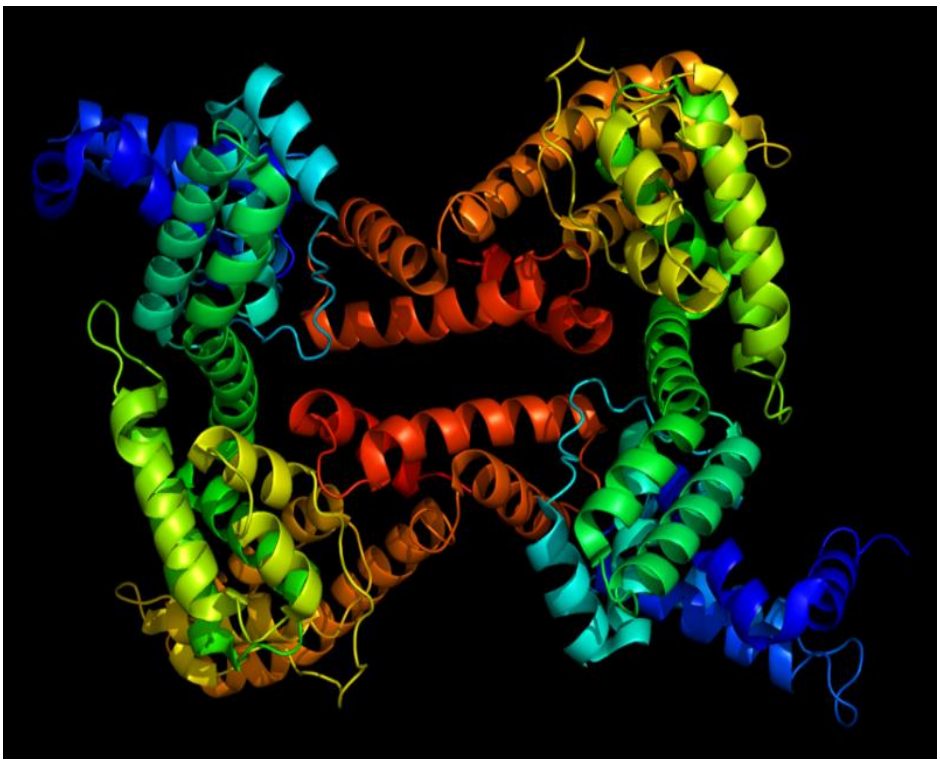

Figure 1-3 Molecular model of vitamin D binding protein.

The structure is composed of a total of 24 alpha helices that make up three domains. The first and second domains are made up of 10 alpha helices each, while the third domain is made up of 4. Source: Wikipedia, copyright-free image.

1.2.3 Endocytic transport – an overview of pathways

The cellular plasma membrane controls the endocytosis and exocytosis of many types of molecules. Endocytosis refers specifically to the uptake or internalization of extracellular components into the cell, while exocytosis refers to the delivery of intracellular components to the plasma membrane and possible secretion (or excretion) of these components from the cell. Through these two tightly regulated transport mechanisms, the cell is able to effectively obtain nutrients from, and react to and influence, its environment. There are several specific endocytic pathways that have been characterized; these include clathrin-mediated endocytosis, caveolar-type endocytosis, CLIC/GEEC-type endocytosis, flotillin-dependent endocytosis, Arf6-dependent endocytosis, circular dorsal ruffles, and the IL2Rβ endocytic pathway (Doherty & McMahon, 2009).

Two more general categories of endocytosis, pinocytosis and phagocytosis, refer to the uptake of solutes and fluid, and uptake of large particles, respectively (Conner & Schmid, 2003). Phagocytosis occurs mainly in specialized mammalian cells. By this process the plasma membrane envelops large particles, and the particles are then internalized as an intracellular phagosome (Conner & Schmid, 2003; Mukherjee, Ghosh, & Maxfield, 1997). Pinocytosis typically refers to at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Conner & Schmid, 2003;

Rodemer & Haucke, 2008). Different ligands and receptors can enter the cell through different endocytotic pathways (Conner & Schmid, 2003).

All of these endocytic events are highly controlled and influence a variety of physiological processes. For example, endocytic transport regulates several important processes including nutrient uptake, cell signalling, cell migration, growth, differentiation and mitosis, pathogen entry, drug delivery, phagocytic cell function and antigen presentation (Doherty & McMahon, 2009). Each of these processes may employ a specific endocytic pathway, but may also have the ability to use different pathways, especially to compensate for the potential loss of one route of entry or uptake.

1.2.3.1 Clathrin-mediated endocytosis

Of all the internalization pathways, clathrin-mediated endocytosis (CME) is best understood. In CME, nutritive and regulatory ligands along with their receptors are endocytosed into clathrin-coated vesicles that bud from phosphatidylinositol 4,5,-bisphosphate (PIP_2)-enriched sites at the plasma membrane and deliver their cargo to the endosomal compartments (Galli & Haucke, 2004; Krauss & Haucke, 2007; Schweizer & Ryan, 2006); ligands or receptors, or some of their components, may be subsequently recycled out of the cell or degraded in the cell. Receptor-mediated uptake of iron-transferrin (Tf), epidermal growth factor (EGF), and low-density lipoprotein (LDL) typically occurs via CME (Rodemer & Haucke, 2008). Clathrin is a heteromeric complex containing three heavy and three light chains, and associates with the membrane

through adaptor proteins; receptors and other integral membrane proteins can also connect to the clathrin lattice via adaptor proteins (Kirchhausen, 2000; Rodemer & Haucke, 2008). Additional, accessory and cytoskeletal proteins can influence CME by, for example, promoting polymerization of clathrin, curvature of the membrane, or fission of the membrane (Rappoport, 2008; Takei, McPherson, Schmid, & De Camilli, 1995; Yarar, Waterman-Storer, & Schmid, 2005). The GTPase dynamin participates in the fission of various types of pits, including clathrin-coated and caveolin-coated pits from the plasma membrane (Bashkirov et al., 2008; Conner & Schmid, 2003; Pucadyil & Schmid, 2008).

Adaptor and accessory proteins can bind the phospholipid PIP_2 that accumulates at CME sites (Beck & Keen, 1991; Ford et al., 2001; Ford et al., 2002; Zoncu et al., 2007). Among the adaptor proteins, the AP-2 complex participates in cargo selection and recruitment of accessory proteins (Robinson & Bonifacino, 2001); there is genetic (Gonzalez-Gaitan & Jackle, 1997; Mitsunari et al., 2005), biochemical (Edeling, Smith, & Owen, 2006; Praefcke et al., 2004), and cytological (Hinrichsen, Harborth, Andrees, Weber, & Ungewickell, 2003; F. Huang, Khvorova, Marshall, & Sorkin, 2004; Motley, Bright, Seaman, & Robinson, 2003) evidence for its central role in CME. Clathrin-coat-associated sorting proteins (CLASPs) include AP-2 and other adaptors that connect the clathrin lattice with integral membrane components and, hence, participate in cargo selection (Haucke, 2006; Lakadamyali, Rust, & Zhuang, 2006; Lewin & Mellman, 1998; Traub, 2005).

1.2.4 Receptor-mediated endocytosis of nutrients and their carrier proteins

Receptor-mediated endocytosis (RME) allows dilute extracellular ligands to be captured and concentrated on pits and in vesicles through the specific action of receptors; non-specific uptake, e.g., of extracellular fluid, does not result in such concentration of specific substances during transport (Alberts et al., 2002; Conner & Schmid, 2003). In RME, the extracellular ligand binds to its complementary membrane receptor, and then the plasma membrane region containing the receptor-ligand complex enters the cell, by CME or other specific endocytic pathways (Alberts et al., 2002; Lodish et al., 2000). The rate at which a particular ligand is internalized depends on several factors including the level of expression of its receptor on the cell surface (Lodish et al., 2000) and the efficiency of the endocytic machinery.

Twenty five or more different receptors are presently known to participate in RME through the clathrin-pathway; and some of these receptors, e.g. Tf and LDL, enter the CME pathway regardless of whether ligand is present or not (Alberts et al., 2002). Other receptors, such as those for growth factors like EGF, are recruited to this pathway only when bound to a specific ligand (Alberts et al., 2002). RME can serve various purposes such as generation of cellular signalling, as with the EGF-EGF receptor (EGFR), and transport of nutrients, as with the transferrin-transferrin receptor (Tf-TfR) (Shankaran, Resat, & Wiley, 2007).

Some of the studies for this thesis project involve nutrient carrier proteins other than DBP. Before discussing endocytosis of DBP (in a separate section below, 1.2.4.1), I will briefly summarize the RME of the two main circulatory

nutrient carriers involved in comparative studies for this thesis project: the iron carrier transferrin (Tf) and the vitamin A carrier retinol-binding protein (RBP).

The existence of cell surface RBP receptors has been suggested by many studies (Blaner, 2007; Bok & Heller, 1976; Creek, Silverman-Jones, & De Luca, 1989; Fortuna, Martucci, Trugo, & Borojevic, 2003; Hagen et al., 1999; Heller, 1975; Hodam, St Hilaire, & Creek, 1991; J. Huang & Vieira, 2006; Kawaguchi et al., 2007; MacDonald, Bok, & Ong, 1990; Mansouri et al., 1998; McGuire, Orgebin-Crist, & Chytil, 1981; Rask & Peterson, 1976; Redondo, Vouropoulou, Evans, & Findlay, 2008; Senoo et al., 1990; Senoo et al., 1993; Sivaprasadarao & Findlay, 1988; Smeland et al., 1995; Sundaram, Sivaprasadarao, DeSousa, & Findlay, 1998; Vahlquist & Torma, 1992). Cells may use different RBP and vitamin A uptake mechanisms, as may be the case with some other vitamins and other regulatory factors (Prasad & Ganapathy, 2000; Ritter, Fajardo, Matsue, Anderson, & Lacey, 1995; A. V. Vieira, White, & Vieira, 1996). Most recently, STRA6 (stimulated by retinoic acid gene 6), a 74 kDa integral membrane protein with several transmembrane domains has been identified as a mediator of retinol uptake from RBP (Blaner, 2007; Kawaguchi et al., 2007; Kawaguchi, Yu, Wiita, Honda, & Sun, 2008; Kawaguchi, Yu, Wiita, Ter-Stepanian, & Sun, 2008; T. White et al., 2008; Wolf, 2007). Mutations in human STRA6 result in various severe pathological phenotypes such as anophthalmia/microphthalmia (A/M) (T. White et al., 2008), mental retardation, congenital heart defects, lung hyperplasia, diaphragmatic hernia, alveolar capillary dysplasia, and others (Golzio et al., 2007;

Kawaguchi, Yu, Wiita, Ter-Stepanian et al., 2008; Pasutto et al., 2007) that are thought to result from the disruption of normal vitamin levels in cells.

Tf is often used as a standard in endocytic transport studies because its receptor-mediated endocytosis is well characterized (Abe, Inoue, Galvez, Klein, & Meyer, 2008; Johnson, Chen, Murchison, Green, & Enns, 2007; Oshiro et al., 1993; Ponka & Lok, 1999). Serum Tf is part of a family of proteins that includes ovotransferrin in oocytes, and lactoferrin in milk (Daniels, Delgado, Rodriguez, Helguera, & Penichet, 2006). The transferrin receptor (TfR, CD71) is an essential protein required for iron uptake, regulation of cell growth and cell survival (Aisen, 2004; Morgan, Smith, & Peters, 1986; Young, Roberts, & Bomford, 1985). Diferric Tf binds to the TfR and both are internalized through clathrin-coated pits (further discussed above).

1.2.4.1 Endocytosis of vitamin D and DBP

DBP has been found to interact with two endocytic receptors, megalin and cubilin; the former has been put forth, with strengthening evidence, as the primary receptor for DBP uptake in some cell types, particularly in the kidney (Christensen & Birn, 2002; Gressner et al., 2008; Nykjaer et al., 1999).

Megalin is a 600 kDa, single-spanning transmembrane glycoprotein and has homology to the low-density lipoprotein receptor family (Christensen & Birn, 2002). This endocytic receptor, located in epithelial cell plasma membranes, has been found in a number of sites throughout the body including the proximal-tubule cells of the kidney (Chatelet, Brianti, Ronco, Roland, & Verroust, 1986a;

Kerjaschki & Farquhar, 1982; Sahali et al., 1988; Seetharam, Levine, Ramasamy, & Alpers, 1988), thryocytes, ependymal cells lining the cerebral ventricles, the epididymus, type II pneumocytes of the lung alveoli (Chatelet, Brianti, Ronco, Roland, & Verroust, 1986b; Kounnas, Haudenschild, Strickland, & Argraves, 1994; Zheng et al., 1994), absorptive intestinal cells (Birn et al., 1997; Levine, Allen, Alpers, & Seetharam, 1984), the placenta (Juhlin et al., 1990; Sahali et al., 1992), oviduct (Zheng et al., 1994), endometrium (Bernadotte, Holmdahl, Juhlin, & Mattsson, 1989; Sahali et al., 1992), visceral yolk sac (Chatelet, Brianti, Ronco, Roland, & Verroust, 1986b; Sahali et al., 1988), mammary cells (Rowling et al., 2006), cells of the inner ear (Kounnas et al., 1994; Mizuta et al., 1999), secretory cells of the parathyroid (Juhlin et al., 1990), and in cilliary epithelium of the eye (Zheng et al., 1994). Megalin expression seems to be preferential to highly differentiated epithelial cells, and can be induced by vitamin D (Liu et al., 1998). Overall, megalin functions in RME and has recently been found to interact with and co-internalize cubilin (Moestrup et al., 1998; Nykjaer et al., 2001). In addition to DBP, a large number of other ligands have been identified for this cell surface receptor including Ca^{2+} (required for megalin-mediated endocytosis of DBP) (Christensen, Gliemann, & Moestrup, 1992), RBP (Christensen et al., 1999), lactoferrin (Willnow, Goldstein, Orth, Brown, & Herz, 1992), apoE and albumin (Cui, Verroust, Moestrup, & Christensen, 1996) as well as many other proteins, lipoproteins, hormones, and enzymes (Christensen & Birn, 2002).

Cubilin is a 460 kDa glycoprotein. Unlike megalin, it is a peripheral membrane protein. Cubilin does not have homology to any other receptor family.

This receptor co-localizes with megalin in the kidney (Chatelet, Brianti, Ronco, Roland, & Verroust, 1986a; Kerjaschki & Farquhar, 1982; Sahali et al., 1988; Seetharam et al., 1988), intestine (Birn et al., 1997; Levine et al., 1984), placenta (Juhlin et al., 1990; Sahali et al., 1992), visceral yolk sac (Chatelet, Brianti, Ronco, Roland, & Verroust, 1986b; Sahali et al., 1988) and in mammary cells (Rowling et al., 2006). Despite also being a multi-ligand receptor, it binds fewer ligands than megalin. Some cubilin ligands include transferrin (Kozyraki et al., 2001), apoA-I and high-density lipoprotein (Hammad et al., 1999). Both cubilin and megalin tightly bind DBP, albeit with slightly different affinities, K_d = 110 ± 15 nM for cubilin and 120 ± 27 nM for megalin (Nykjaer et al., 2001).

After filtration of the DBP complex within the renal glomeruli, both megalin and cubilin allow for the (re)uptake of calcidiol-DBP in the proximal-tubules (Christensen & Birn, 2002). This is followed by metabolite activation of calcidiol to 1-calcitriol in the renal mitochondria (Christensen & Birn, 2002; Rowling et al., 2006). Nykjaer et al. (2001) provided evidence that both megalin and cubilin work together to facilitate the endocytic process and that the lack of one or the other halts or considerably reduces the uptake of DBP and its ligand.

Although megalin is the primary endocytic receptor, the DBP complex binds cubilin in a Ca^{2+}-dependent manner, which anchors it to the cell surface and enhances megalin-mediated endocytosis of the DBP-calcidiol-cubilin complex. Accordingly, *in vitro* studies show that inhibition of cubilin by the addition of an anti-cubilin antibody reduces DBP uptake by approximately 70%, while the addition of RAP (receptor-associated protein), which blocks all ligand-binding sites

on megalin (including those of cubilin), completely impedes DBP uptake (Nykjaer et al., 2001). Gressner et al. (2008) have shown that the inhibition of megalin by anti-megalin antibody is further intensified with EDTA, a metal ion scavenger, providing support to the Ca^{2+}-dependent action of megalin-mediated endocytosis. On the other hand, Rowling et al. (2006) found that human mammary epithelial (HME) cells and T-47D breast cancer cells, both of which co-express the two receptors, are readily able to endocytose calcidiol-DBP, whereas MCF-7 breast cancer cells, in which only megalin expression was detected, are unable to internalize the DBP complex. Megalin-deficient mice and individuals with genetic mutations in cubilin have much lower plasma levels of both calcidiol and calcitriol (Nykjaer et al., 1999; Nykjaer et al., 2001).

Most of these cubilin-megalin ligands (e.g., RBP, Tf, HDL) also have other, more specific receptors. Thus, for a carrier protein such as DBP, it is possible that there are different receptors—including ones more specific than megalin and cubilin—and transport pathways in different tissues or even within the same cell type.

Subsequent to internalization of the protein carrier-ligand complex, the vitamin D receptor (VDR) pathway can become activated. This is mediated by the presence of the ligand, 1-calcitriol or calcidiol (calcidiol is converted to 1-calcitriol by CYP27B1), which induces expression of a variety of genes including the well established expression of CYP24, a VDR reporter gene (Rowling et al., 2006).

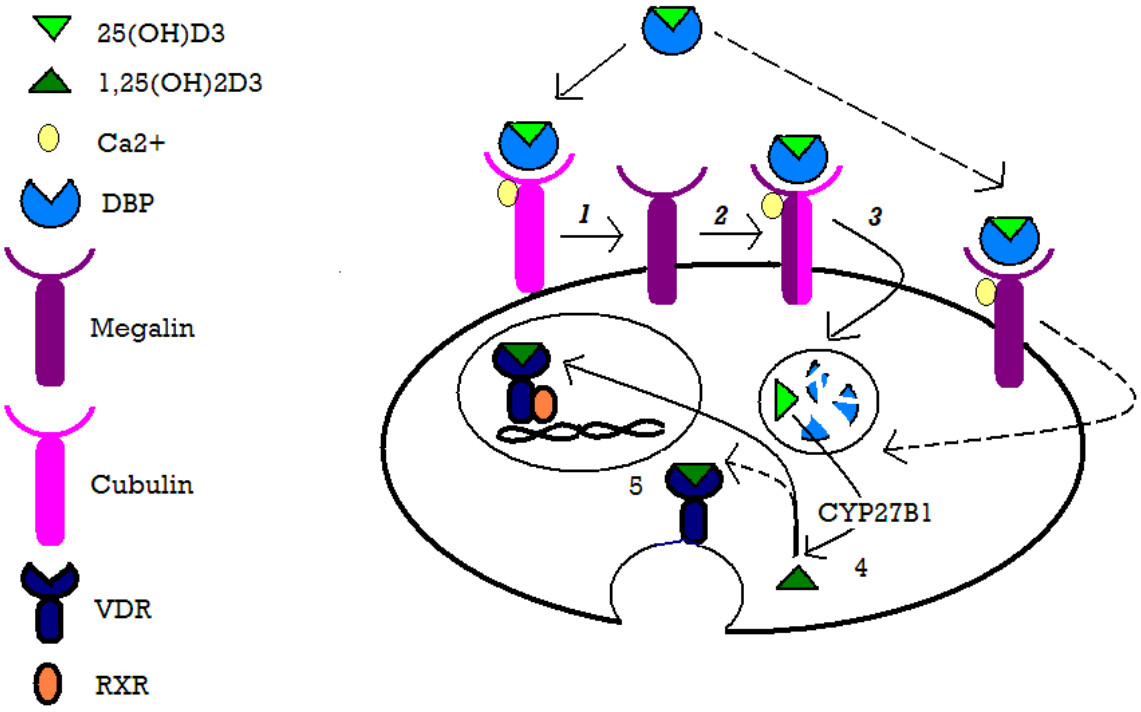

Figure 1-4 Schematic of megalin-cubilin-mediated endocytosis of DBP.

DBP binds circulating 25(OH)D3 and the calcidiol-bound complex is sequestered to the cell surface by cubilin in a Ca^{2+} dependent manner (1). Megalin then interacts with (2), binds and internalizes (3) the calcidiol-DBP-cubilin complex. Alternatively, megalin can bind directly to calcidiol-DBP. After endocytosis, CYP27B1 converts the internalized metabolite to $1,25(OH)_2D3$ (4) which then binds nuclear-VDR or caveolae-associated VDR to induce genomic or non-genomic responses, respectively (5). This figure is based on Figure 5 of Nykjaer et al. (2001).

1.3 Main hypothesis tested

The following are the main hypotheses that have been tested in relation to the above objectives:

(i) DBP receptors capable of mediating DBP endocytosis exist on human epidermoid A431 cells and murine hepatocytes.

(ii) DBP endocytic transport is influenced by cell growth status and exposure to growth modulators such as epidermal growth factor (EGF).

(iii) Gender differences exist with regard to DBP endocytosis, and these differences correlate with the effect of sex hormones on DBP endocytic transport in isolated cells.

(iv) Membrane preparations from different mouse tissues differ in terms of total DBP binding capacity.

(v) DBP membrane binding capacity is influenced by aging.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and biochemical reagents

Vitamin D-binding protein (DBP) and anti-DBP IgG was obtained from Abcam (Massachusetts, U.S.). Vitamin D3 (cholecalciferol), human epidermal growth factor (EGF), Human holo-transferrin (Tf), biotinylated Tf (bTf), streptavidin-peroxidase (ESa or streptavidin-HRP), peroxidase (HRP), *o*-phenylenediamine dihydrochloride, biotin N-hydroxy- succinimide (BNHS), human retinol-binding protein (RBP), anti-RBP IgG, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Ontario, Canada). Protease inhibitor pellets were purchased from Roche. Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum (FBS) were obtained from different sources: Invitrogen (Ontario, Canada), Gibco (Ontario, Canada), and Sigma-Aldrich (Ontario, Canada). Phosphate-buffered saline (PBS) was obtained from Gibco (Ontario, Canada).

The ligand-enzyme conjugates (ESa-bX, X = protein ligand such as Tf or DBP or RBP) were prepared by incubating ESa with bX at a 1:1 molar ratio in PBS

for 1 h at room temperature, typical incubation conditions previously reported (Graf & Friedl, 1999; Kamemura & Kato, 1998; Shimada, Mernaugh, & Guengerich, 2005; A. Vieira, 1998; Weitz-Schmidt et al., 1996), for the formation of stable ESa-bX complexes. Preparation of b-DBP as well as other methods, chemical reagents, and equipment are detailed below in the relevant sections.

2.2 Cells, cell preparations and animals

Murine liver cells were isolated from male and female animals using a non-enzymatic, EDTA-sucrose-based method. This novel, simplified method was based on previously reported well-characterized EDTA-sucrose procedures (Kravchenko, Petrenko, Shanina, & Fuller, 2002), and is detailed in the Appendix.

The human A431 epidermoid cell line was purchased from ATCC (American Type Culture Collection) and cultured in DMEM containing 10% FBS, incubator set to 37°C, 5% CO_2. For the endocytosis experiments (section 2.3.2 below), cells were cultured in multi-well plates (Sarstedt).

For the preparation of semi-intact cell membranes (SICM), A431 cells were grown in DMEM (Gibco) supplemented with 10% FBS (Sigma-Aldrich) in a humidified incubator (VWR), 5% CO_2, 37°C. Cells were grown to a confluency of approximately 80-90%, and then incubated for 30 min in serum-free medium. They were then gently washed with 1ml ice-cold PBS and 1ml ice-cold KSHM (100 mM potassium acetate, 85 mM sucrose, 20 mM HEPES and 1 mM magnesium acetate). Cells were then scraped with a rubber tool and collected in

an Eppendorf tube kept on ice. One hundred to 200 µl KSHM was added twice to the plate, each time scrapping to collect any leftover cells. The Eppendorf tube (kept on ice) was centrifuged for 1 min at 12,050 *x g*, and the supernatant was removed. Subsequently, the cell pellet was resuspended in an equal volume (typically 100-200 µl) of high sucrose-KSHM (100 mM potassium acetate, 0.75 M sucrose, 20 mM HEPES and 1 mM magnesium acetate) and stored frozen at -80°C. For binding assays, equal volumes of SICM were aliquoted into tubes, each aliquot removed immediately after resuspension to ensure equal membrane amounts.

2.2.1 Animals

The animal-based work has been approved by the Simon Fraser University Animal Care Committee. For the animal injection and tissue preparation experiments, C57BL/6 middle-aged male and female mice were used, approximately 5 to 7 months of age (Charles River Laboratories, USA). The animals were injected with biotin-labelled DBP dissolved in PBS into the tail vein, 100 µL maximum injected volume. Control injection experiments without ligand (PBS solution) were also performed. Before organ/tissue dissection, the euthanized animals were perfused with physiological saline (see also Appendix).

2.3 Major Methods

2.3.1 Ligand biotinylation

Biotinylated DBP (b-DBP) was prepared by incubating biotinylation reagent, BNHS (Biotin N-hydroxysuccinimide ester; Sigma) with DBP at a molar ratio of 5:1, biotin:protein. The reagents were mixed in an Eppendorf tube, vortexed, and incubated for 30 min at room temperature. TBS was then added and the solution aliquoted and stored -80°C. A similar procedure was used for the preparation of b-RBP and b-Tf (the latter was also purchased from Sigma-Aldrich). Biotinylated proteins were detected by ELISA procedures (e.g., section 2.3.2) using streptavidin-HRP.

2.3.2 Endocytosis assays

For testing effects of different cell growth densities, cells were cultured on 6 well plates to be approximately 25%, 50% and 90% confluent on the day of the experiment. Prior to analysis of endocytosis, cells were incubated in serum-free medium (SFM) for 4 h, rinsed 2 times with PBS, and incubated with (i) ESa-b-DBP (ligand-enzyme conjugate, 4.0 µg/ml) and (ii) Esa-b-Tf (comparative control, 5 µg/ml) in PBS-SFM (a 1**:**1 volume mixture of PBS, and serum-free DMEM) for 10 min at 37°C. Cells were washed 2 times with ice-cold acidic buffer (25 mM acetic acid and 100 mM NaCl, pH 3), followed by neutralization with PBS, and addition of detergent solution (1% Triton X-100, 0.1% SDS in 1 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.4) to the cells. Cell lysates were analyzed on 96-well plates

and internalization of conjugates was assessed through spectrophotometric measurements of a peroxidise(E)-catalyzed colour-generating reaction. This reaction involves 0.4 mg/ml *o*-phenylenediamine dihydrochloride (OPD) and 0.02% hydrogen peroxide in 50 mM Na_2PO_4 and 30 mM sodium citrate, pH 5. The colour reactions were terminated with 2 N sulfuric acid. Absorbance was measured at 492 nm (Statfax-2100 ELISA plate reader).

An alternative method was also used for the endocytosis assays. The biotinylated ligand, b-DBP (or b-Tf, b-RBP) was used; and ultimately endocytosed ligand was captured on anti-ligand coated wells (Section 2.3.3). The procedure was similar to that described above: cells were cultured to 25%, 50% and 90% confluencies and, after 4 h in SFM, incubated with (i) b-DBP (4.0 µg/ml) and (ii) b-Tf (comparative control, 2.5 µg/ml). After incubation, cells were again treated as above; however, cells lysates were transferred to 96-well anti-ligand antibody coated plates (e.g., anti-DBP IgG) that had been previously blocked with 0.2% BSA in PBS solution and rinsed with PBS-Tween (e.g., section 2.3.3). Lysates were then incubated on the plates for at least 16-20 h at 4°C. Lysates were then removed, and the wells blocked with BSA, rinsed with PBS-Tween and incubated with 0.001 µg/µl streptavidin-HRP (STAV-HRP) in PBS for 4 h at 37°C. Cells were then rinsed with PBS-Tween 3 times before spectrophotometric-based assessment of internalization (same as above).

For testing potential inhibitory or stimulatory activities of various factors (growth factors, hormones and other chemical compounds), the substances were added at the concentrations indicated (Section 5) either immediately before the

biotin-protein ligand or for a prolonged (typically, 30 min) incubation before addition of biotin-protein ligand. In these cases, cell growth density was approximately 50%, unless noted otherwise in the figure legends and results sections.

2.3.3 Preparation of antibody-coated plates

For preparation of antibody-coated plates, wells were coated with 200 µl of anti-DBP (4 µg/ml; or other anti-ligand antibodies at the same concentrations) in 0.05 M carbonate/bicarbonate buffer at pH 9.6 and incubated for at least 24 h at 4°C. Non-bound antibody was then removed and wells were blocked with 200 µl 0.2% BSA in PBS for at least 2 h at room temperature. After removal of the blocking solution and a rinse with PBS-Tween, wells were ready for addition of lysate.

2.3.4 Mouse tissue analysis for b-DBP

Kidney, lung, adipose, brain, and liver tissues from C57BL/6 mice injected with b-DBP, ESa-b-DBP, ESa (control), and PBS alone (control), were homogenized on ice in extraction buffer (EB: 0.5×PBS [10 mM phosphate, 75 mM NaCl, pH 7.4], 0.1% Triton X-100, 0.01% SDS and protease inhibitors) at a ratio of 2 ml EB per gram of tissue. Tissue extracts were centrifuged at 12,050 *x g* for 15 min at 4°C. The resulting supernatants from ESa or Esa-b-DBP injected tissues were removed and loaded onto a 96-well plate (Nunc), after which the colour reaction was performed. The supernatants from the b-DBP and PBS injected

tissues were loaded onto a 96-well plate (Nunc) pre-coated with 4 µg/ml anti-DBP IgG in 50 mM sodium bicarbonate buffer (pH 9.6), and then incubated 16-20 h at 4°C. The wells were then rinsed, incubated with streptavidin-HRP, rinsed again, and the OPD colour reaction was performed as detailed in Section 2.3.2.

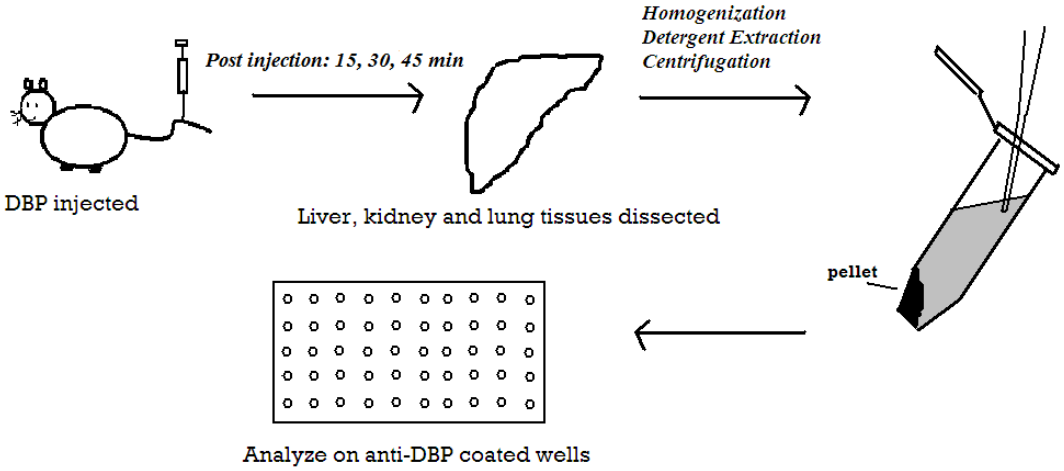

Figure 2-1 Flowchart of tissue accumulation analysis procedure.

Biotinylated DBP (b-DBP) is injected into the tail vein of the mouse. At the time points of 15-45 min post-injection, liver, kidney, lung and other tissues are dissected out, extracted with detergent and analyzed for b-DBP on wells coated with anti-DBP IgG.

2.3.5 Analysis of DBP binding to crude membranes prepared from tissues and A431 membranes

Tissue samples from non-injected animals, middle-aged (5-7 months) male and female mice, and young (1-2 months) and old (25-27 months) male mice, were homogenized as described above for the injected tissues. After homogenate centrifugation the supernatant was discarded and the pellet re-suspended in 2 volumes of PBS. Bovine serum albumin (BSA) was added to give a final BSA concentration of 0.02%. The resulting crude extract was divided into 3 samples of equal volume. To keep the samples as homogenous as possible, vortexing of the tube was performed before the removal of each sample volume. Then 2 μg of biotinylated DBP (b-DBP) was added to each sample and the samples were incubated for 16-20 h at 4°C with gentle mixing. The extracts were centrifuged at 12,050 *x g* (Spectrafuge 16M) for 5 min at 4°C, followed by re-suspension of the pellet with a PBS solution containing 0.02% BSA. The extract was re-centrifuged, and the resulting pellet rinsed with PBS and then re-suspended with (ST) blocking buffer (pH 7.4). This final extract was plated onto a 96-well plate (Nunc) pre-coated with 4 μg/ml anti-DBP IgG (Section 2.3.3), and then incubated 16-20 h at 4°C. After rinses and streptavidin-HRP probing as above, an OPD colour reaction (see Section 2.3.2 for details) was performed to assess the captured b-DBP. Binding analyses on A431 membranes (SICM; Section 2.2) was performed using the same procedure as the one described above.

2.3.6 Analyses of DBP binding to cells

Cells were prepared as described above for the endocytosis assays (Section 2.3.2), but the ligand was added to the cells on ice and the binding incubation was performed in a 4°C environment (to prevent ligand internalization, cells and ligand were never warmed above 4°C). After incubation with ligand for 16-20 h, cells were rinsed twice with PBS-BSA as above, and a detergent solution (1% Triton X-100, 0.1% SDS in 1 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.4) was added to lyse the cells. Lysates were then analyzed on anti-DBP-coated plates as described in section 2.3.2.

2.4 Statistics

Results are presented as the group mean ± standard error of the mean (SEM) for each experimental group, unless noted otherwise. The number of measurements, *n*, is indicated in the figure legends. Two-tailed (unless noted otherwise) t-tests were performed on the data to compare a given experimental treatment with control. Two p-value levels—$p<0.05$ but greater than or equal to 0.01, and $p<0.01$—are indicated in the figure legends with * for statistical significance (alpha level, 0.05).

CHAPTER 3: RECEPTOR-MEDIATED ENDOCYTIC TRANSPORT OF DBP AND OTHER NUTRIENT CARRIER PROTEINS

3.1 Introduction

The potential mechanisms by which DBP mediates cellular uptake of vitamin D and its hydroxylated derivatives are not well defined (cf. Section 1.2.4.1). Analysis of DBP transport is of fundamental importance in terms of studying these mechanisms. Experiments have been performed in an attempt to further characterize DBP receptor-mediated endocytic transport in an epithelial line, human A431 epidermoid cells. Epithelial cells are considered to be good models for DBP transport studies (cf.(Ternes & Rowling, 2009); see also Section 5.1); and epidermal cells are known to produce biologically active vitamin D metabolites (Hall et al., 2010; Holick, Smith, & Pincus, 1987; MacLaughlin, Anderson, & Holick, 1982). Characterization of possible DBP receptors and DBP endocytosis, however, has not previously been reported for epidermal cells or for

the A431 cells. The results of my work have provided novel information about DBP and these cells. Moreover, the RME of other nutrient carrier proteins including RBP and Tf has previously been investigated using such cells (references in Section 1.2.4), and provides a valuable comparison.

Basic knowledge obtained from these studies has provided novel insight into the physiological transport of DBP. Such knowledge forms a necessary basis for studying modulation of DBP in other physiological and pathological contexts (discussed further in Section 5.1). Moreover, an understanding of DBP receptor-mediated endocytosis may be useful for DBP-mediated delivery of therapeutics to specific cells (cf. RBP-mediated delivery of therapeutics in Section 5.1).

3.2 Results and Discussion

A431 cells are known to express Tf and RBP receptors at the cell membrane (Castagnola, MacLeod, Sunada, Mendelsohn, & Taetle, 1987; Hopkins & Trowbridge, 1983; J. Huang & Vieira, 2006; A. Vieira, 1998; Wiley, 1988). Both the RBP and Tf receptors lead to internalization of their respective nutrient-carrier ligands in these cells; and, at least in the case of Tf, the clathrin-mediated endocytic pathway is established (J. Huang & Vieira, 2006) and references therein, (Azizi & Wahl, 1997; Hopkins & Trowbridge, 1983; Wiley, 1988). In this context, it was of interest to examine and compare receptor binding activities and the endocytic pathway(s) of DBP, another nutrient carrier protein.

A rough estimate of DBP-binding activity on A431 cells was obtained by comparing total Tf and DBP binding using two different methods: (a) binding to intact cells and (b) binding to isolated cell membrane fractions. Figure 3-1 shows that in both cases the total DBP-binding activity was greater than that of Tf in these cells, and similar results were obtained with both methods. Tf is known to have a B_{max} of 1 x 10^5 binding sites per A431 cell. With future work to define the non-specific binding activity of DBP, and the K_d for the DBP-Receptor interaction, a B_{max} for DBP binding to these cells may be calculated. In comparison, total RBP binding in these cells is lower than that of Tf; and the RBP B_{max} has been calculated to be lower than that of Tf (Huang and Vieira, 2006).

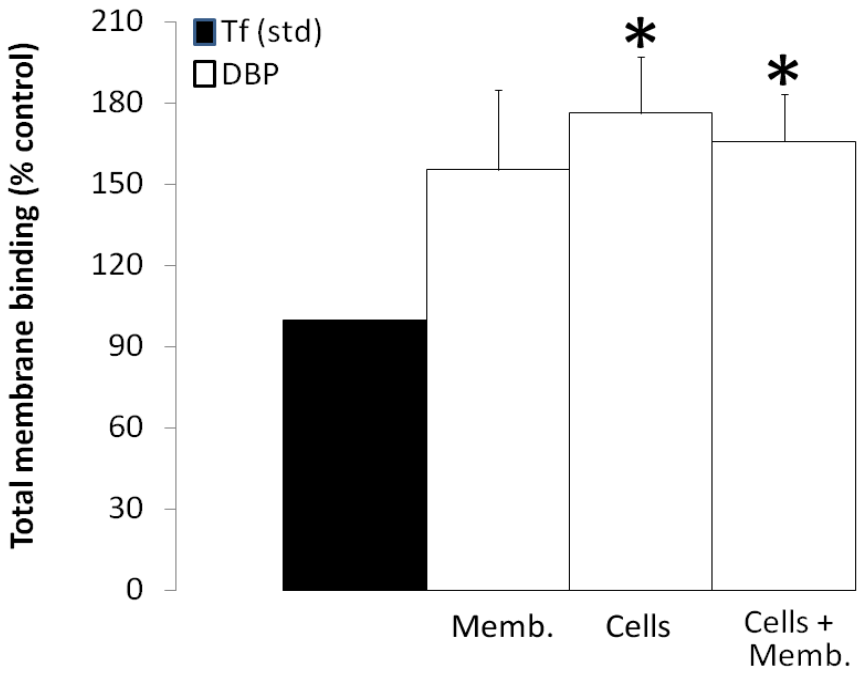

Figure 3-1 Comparison of total Tf and DBP binding to A431 cells and membranes.

A431 cells or membrane fractions were incubated with Tf (control) or DBP ligands at 4°C (to avoid endocytosis) as described in Materials and Methods. A431 cells appear to have a significantly higher relative total binding activity when compared to Tf (asterisks, p<0.05). "Cells + Memb." represents a composite of both data sets. (n = 6; replicates = 3 for all cases)

Comparisons of total DBP binding activities in membrane preparation of various mouse tissues were also performed (non-specific binding was not measured; so only total membrane binding activity of the membrane preparations is discussed). Liver, lung, adipose, kidney and brain tissues from both male and female mice were homogenized and incubated with labelled-DBP as described in section 2.3.5 of Materials and Methods. As shown in Figure 3-2, the presence of receptors for DBP is suggested in all of the above named tissues, in agreement with previous studies (Christensen & Birn, 2002; Jones et al., 1998; Ochs-Balcom et al., 2011). Crude membrane preparations of liver tissue have the highest relative binding activity, followed by the kidney, while membrane preparations from lung and adipose tissue bind 5 to 10 times less DBP, respectively, when compared to the liver. The binding activities of all tissues are significantly different when compared to the liver (lung, adipose, brain, $p<0.01$; kidney, $p=0.02$). The liver is the primary site for DBP synthesis and secretion, and both the liver and the kidneys are key activation sites for biologically active calciferols (P. White & Cooke, 2000). If one assumes the presence of the same receptor(s) in these tissues and similar non-specific binding, the observed differences in binding activity among the tissues may relate directly to receptor abundance.

To provide a more relevant context for possible estimates of endocytic efficiency, tissue DBP accumulation data were corrected for total tissue DBP binding. Figure 3-3 suggests that DBP receptors in the kidney are significantly more efficient than in any of the other tissue. This result coincides with the kidney being the major site at which the dominant circulating metabolite gets converted to

the major active metabolite, in the pathway of vitamin D3 activation. At this point, the reason for the relative difference between b-DBP binding (Fig. 3-2) and endocytic efficiency (Fig. 3-3) in liver tissues is not clear. It may be that the liver contains a large number of DBP receptors that are not highly functional in terms of endocytosis. Indeed, the liver seems to have some unique endocytosis-related properties compared to other tissues (Keyel et al., 2006). It is also possible that the liver endocytoses more DBP compared to the kidney, but a relatively greater percentage of it is degraded or recycled in the time (~20 min) between injection and removal of tissues.

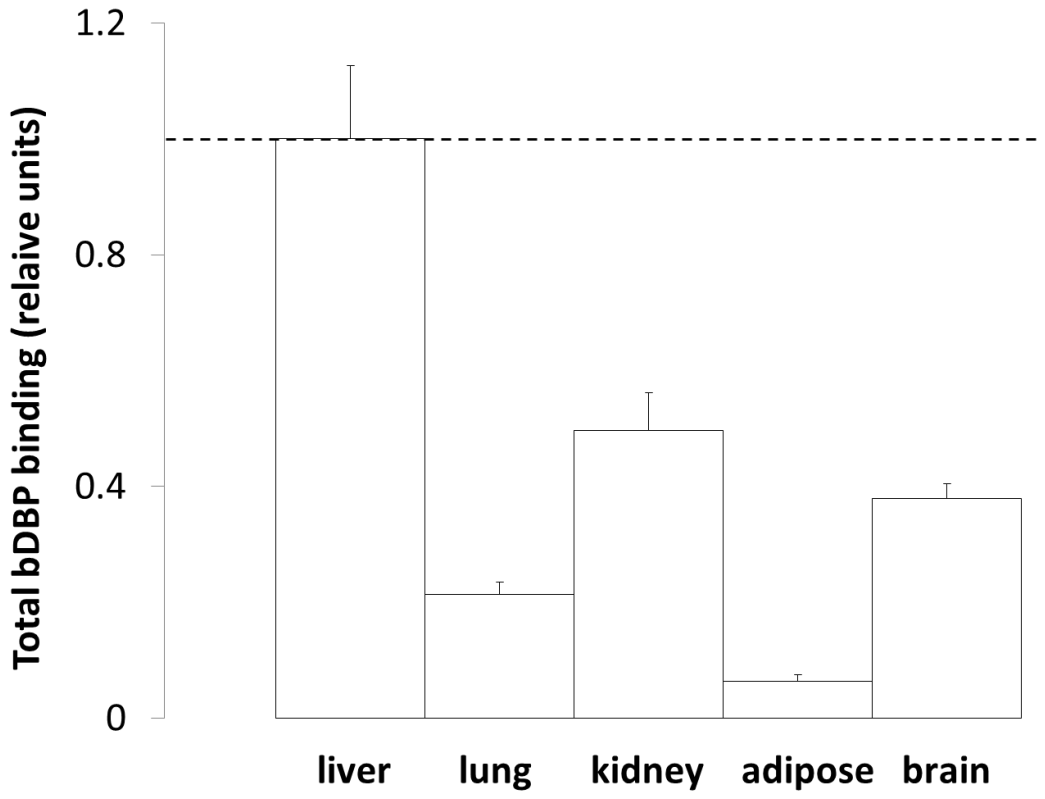

Figure 3-2 Comparison of total DBP binding capacities in crude membrane preparations from various mouse tissues

The liver, lung, kidney, adipose and brain tissues from both male and female mice were homogenized to obtain crude membrane samples and incubated with labelled-DBP at 4°C as described in Materials and Methods. Tissues from male and female animals combined (the same relative pattern of binding is observed in male and female tissues) show the liver and kidney have the highest relative DBP binding capacity, followed by the brain, lung and adipose, respectively. The binding capacities of all tissues are significantly different when compared to the liver (all tissues, $p<0.01$). All values are corrected for with the use of PBS controls (no b-DBP). (n = 9; replicates = 3 for each tissue for both male and female)

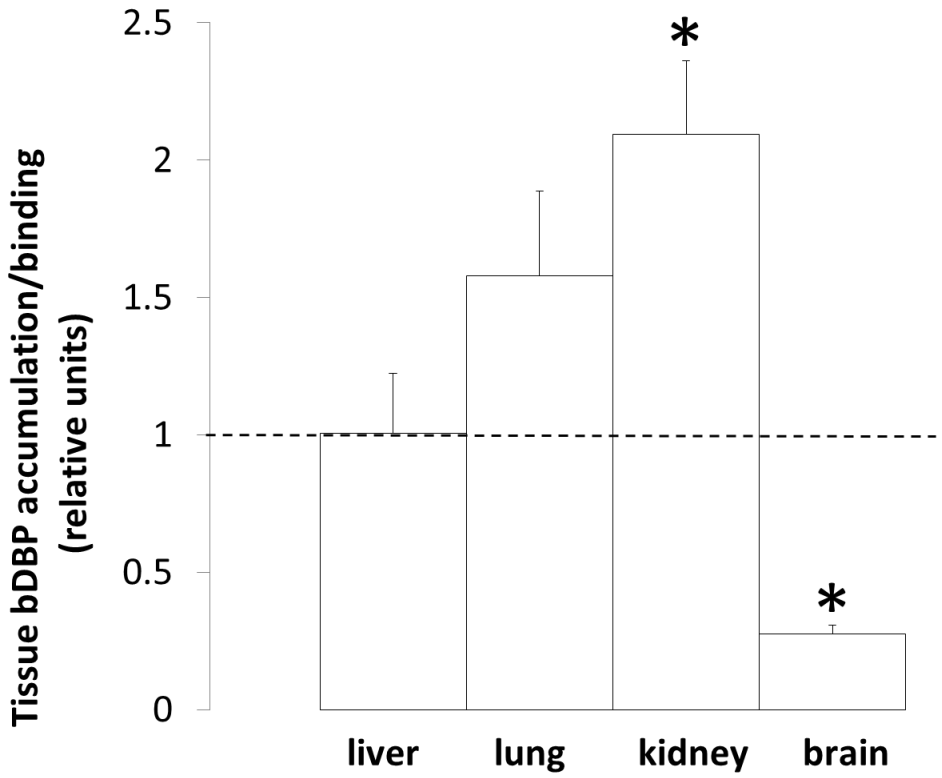

Figure 3-3 Comparison of accumulation to binding ratios of different mouse tissues

Accumulation data were divided by the respective binding data. Data from male and female tissues were combined (the same relative pattern of binding is observed in male and female tissues) and comparisons between tissues show that the DBP receptors in the kidney are significantly more efficient when compared to the liver ($p<0.01$), while DBP receptors in the brain are significantly less efficient ($p=0.01$). It should be noted that the adipose tissue data (not shown) have very high variability. ($n = 8$ for all tissues; replicates = 2 for each male and female)

In terms of the endocytic pathway taken by DBP, chemical inhibitors of two known pathways were tested as described in Materials and Methods. Chlorpromazine is an inhibitor of the clathrin-dependent pathway, that acts upon adapter protein-2 (AP-2) affecting its binding ability and thus the recycling and assembly of the clathrin coat on the plasma membrane (Bhattacharyya et al., 2010; L. H. Wang, Rothberg, & Anderson, 1993). Filipin, on the other hand, binds to cholesterol- and sphingomyelin-rich cell membrane domains (rafts) and is a known inhibitor of the caveolar (caveolin-dependent) endocytic pathway (Kang, Zhao, Lovaas, Eisenberg, & Greene, 2009; Rothberg et al., 1992). As shown in Figure 3-5, when testing the effects of these two modulators on DBP endocytosis in hepatocytes, chlorpromazine has no statistically significant effect on the amount of labelled-DBP accumulation in the cells. The addition of filipin, however, does significantly ($p<0.05$) decrease the amount of DBP endocytosed when compared to the control. This inhibition by filipin suggests that DBP endocytosis in hepatic cells may be caveolin-dependent. In astrocytes, megalin-mediated transport through the caveolar endocytic pathway has been reported ((Bento-Abreu et al., 2009); the megalin receptor, however, can also function through the clathrin-mediated pathway in some cell types (Fleming, Mar, Franquinho, Saraiva, & Sousa, 2009; Lambot et al., 2006). β-estradiol, a sex hormone also tested for its modulation on DBP internalization, has no significant affect on these male hepatocytes; but as presented in Chapter 5, estradiol does have a major effect on female hepatocytes.

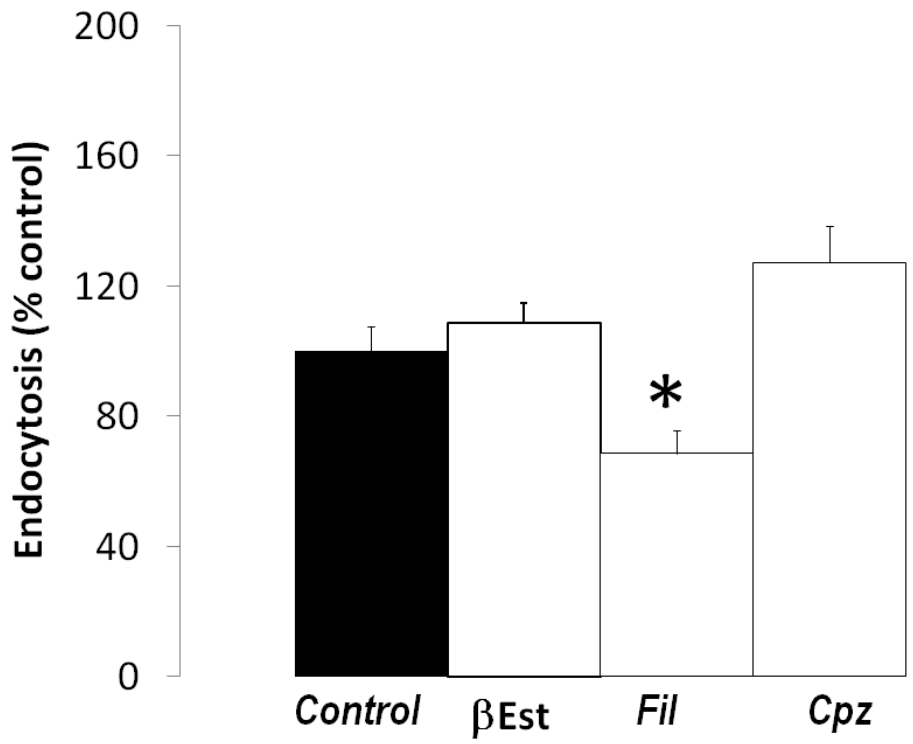

Figure 3-4 Comparison of the effects of different endocytic modulators on DBP internalization by hepatocytes.

The relative accumulation of DBP after a 10 min endocytosis period was assessed in cells treated with chlorpromazine and filipin known inhibitors of clathrin- and caveolin-dependent endocytic pathways, respectively. When compared to the control, both inhibitors appear to affect the amount of DBP internalized. Addition of chlorpromazine increases DBP accumulation ($p>0.05$); filipin, however, significantly decreases ($p<0.05$) DBP accumulation relative to the control. The sex hormone β-estradiol causes only a non-statistically significant increase in these male hepatocytes ($p>0.05$). ($n = 4$; replicates = 2 in all cases)

CHAPTER 4: GENDER AND AGING EFFECTS ON DBP TISSUE ACCUMULATION AND MEMBRANE BINDING

4.1 Introduction

Differences in Vitamin D levels can be observed, not only with age, skin type and geographical location, but, in some cases, also with regard to gender (Hagenau et al., 2009; Smith, 2010). Whether Vitamin D serum levels are higher in males or females, however, still remains controversial. Hagenau et al. (2009) completed a meta-analysis on a multitude of studies and found that serum calcidiol levels were significantly higher in females (56 ± 1.6 nM) than in their male counterparts (50 ± 2.6 nM). In contrast, when completing a longitudinal study of individuals from Southern Italy, it was found that men had significantly higher levels of serum calcidiol in comparison to women (Carnevale et al., 2001). Studying serum calcitriol levels in a local population (Peterborough, Ontario) Smith (2010) found significant differences only between South Asian men and

women, but no difference in Caucasians. The sex differences in DBP and vitamin levels warrant further research.

In the experiments detailed below, tissue accumulation of injected b-DBP was analyzed in the tissues of male and female mice. Such tissue accumulation does not provide direct indications of RME and endocytic efficiency in the cells; but early post-injection time points may provide an estimate of such transport. Experiments directed at analyzing possible differences in DBP-binding efficiencies with gender were also performed. The combination of the binding and accumulation data may, thus, allow the estimation of endocytic efficiencies.

Aging is known to affect the efficiency of various physiological transport processes including endocytic trafficking and receptor-mediated endocytosis (RME) (Cessac, Perichon, Schaeverbeke, & Bakala, 1993; Cnop et al., 2000; Dini, Rossi, Marchese, Ruzittu, & Rotilio, 1996; Haq & Szewczuk, 1992; Ito et al., 2007; Le Couteur et al., 2008; Park et al., 2002; Park, 2002; Verbeke, Perichon, Schaeverbeke, & Bakala, 1996) and references therein). Blanpied et al. (2004) for example, have shown that aging decreases the efficiency of Tf endocytosis (CME). There is evidence for decreased rates of Tf RME in aged animals (Chen, 2009; Morgan & Moos, 2002). Possible effects of aging on RME of DBP and uptake of calciferols are not known. We were not able to obtain young and old mice to directly address this issue; however, we were able to use frozen tissues from young and old mice to prepare membrane fractions and analyze DBP binding.

4.2 Results and Discussion

In order to discuss possible gender-related differences in DBP transport to cells and tissues, a related analysis of the total DBP binding capacity of the tissues or cells is required. Differences in accumulation, for example, may not be due to differences in endocytic efficiency, but may be due instead to the relative abundance of membrane receptors (or to different DBP degradation rates). By calculating the ratios of 15-20 min accumulation to total binding, one may estimate relative endocytic efficiency (rates of uptake). This estimate requires the assumption, however, that possible differences in other biochemical events (e.g., b-DBP degradation) within the cells over this time period are negligible.

Figure 4-1 shows tissue accumulation levels between 30-45 min after injection of a HRP-streptavidin-b-DBP protein complex (stav-DBP) into the circulation of male and female mice. Tissues from the mice were collected and analysed against control tissues (injected with HRP-streptavidin only) as described in Materials and Methods. The results from the tissue analyses show that, although there does not seem to be differences in stav-DBP accumulation between male and female kidney tissues, a significant difference ($p<0.05$) in accumulation is found between female adipose tissue when compared to the amount of stav-DBP in male adipose. As well, there is a significant difference ($p<0.05$) between male and female adipose tissues when compared to male and female kidney tissues, respectively. Male liver tissues appear to accumulate less

stav-DBP than female liver tissues; this difference is statistically significant ($p<0.05$) only with a one-tailed t-test.

Figure 4-2 shows tissue accumulation to binding ratios between 15-20 min after injection of a biotin-labelled DBP (b-DBP) into male and female mice. Tissues from the mice were collected and analysed as described in the Materials and Methods. Similar to what was found with the stav-DBP data, the male:female ratios illustrate that receptors for DBP in liver are significantly less efficient in males than in females ($p<0.05$), while no significant differences in DBP endocytic efficiency are found between the other male and female tissues examined. The efficiency of DBP internalization is approximately 1.5 times higher (but $p>0.05$, no statistical significance) in male kidney tissues than in females. It was noted during tissue collection that many of the male mice possessed only 1 large kidney, whereas the females possessed two smaller ones. This relatively large male:female kidney ratio could be influenced by the morphological asymmetry; for example, functional overcompensation by the male kidneys may involve an increase of receptors on the cell surface or an increase in efficiency of the endocytic process.

Figure 4-3 shows cellular b-DBP and b-RBP accumulation levels after a 10 min endocytosis period in isolated male and female hepatocytes as described in the Appendix. The figure illustrates a significantly lower accumulation of b-DBP in male compared to female cells. But this is not the case for RBP. Perhaps there are gender-related differences for the two receptors or the two endocytic pathways. The significantly lower accumulation of b-DBP in male hepatocoytes as compared

to female cells is in agreement with the accumulation data from both stav-DBP and b-DBP in male and female liver tissues (Chapter 3). One possible explanation is that female hepatocytes have a higher level, or activity, of one of the many factors that can influence endocytic efficiency.

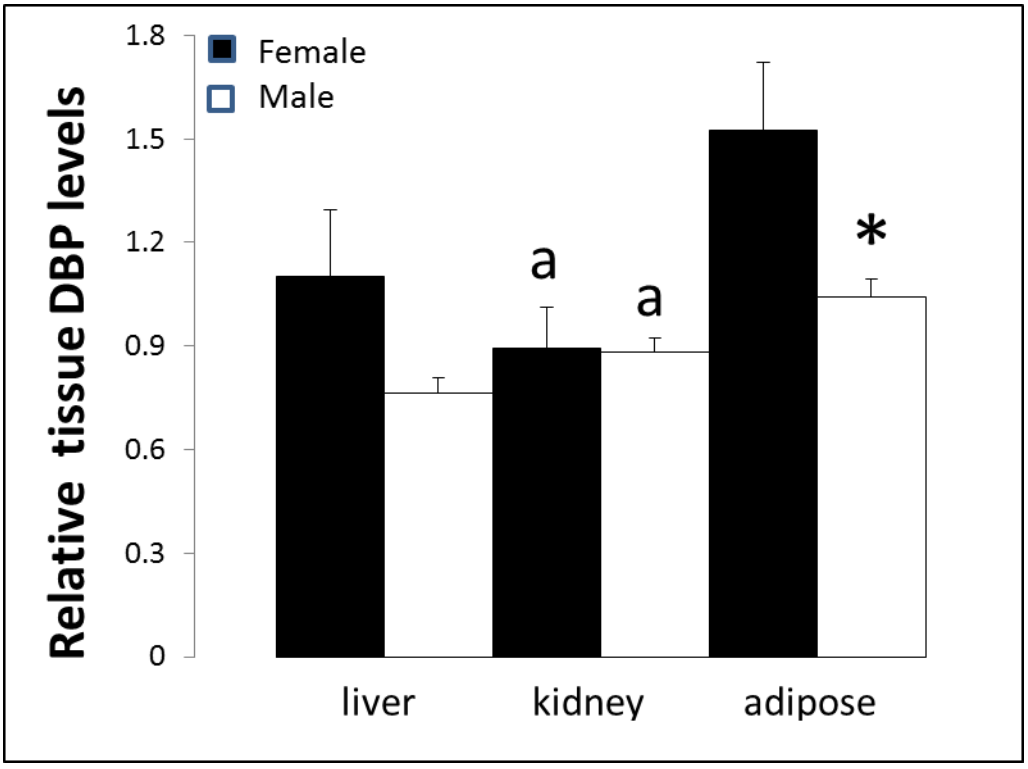

Figure 4-1 Comparison of stav-DBP accumulation levels in male and female tissues.

The accumulation of streptavidin-DBP protein complex in both male and female liver, kidney and adipose tissues is shown after a 30-40 min post-injection time point. The amount of stav-DBP accumulation in female adipose tissue is significantly greater than in male adipose ($p<0.05$, asterisk). And both males and females accumulate more in adipose than in the respective kidney tissues (a, $p<0.05$). No significant differences are found between male and female kidney tissues. Lower accumulation in male liver tissue relative to female liver tissue is observed and is significant with a one-tailed test ($p<0.05$). All values were corrected for with the use of streptavidin only controls (no DBP ligand). (n = 6; replicates = 3 for all tissues)

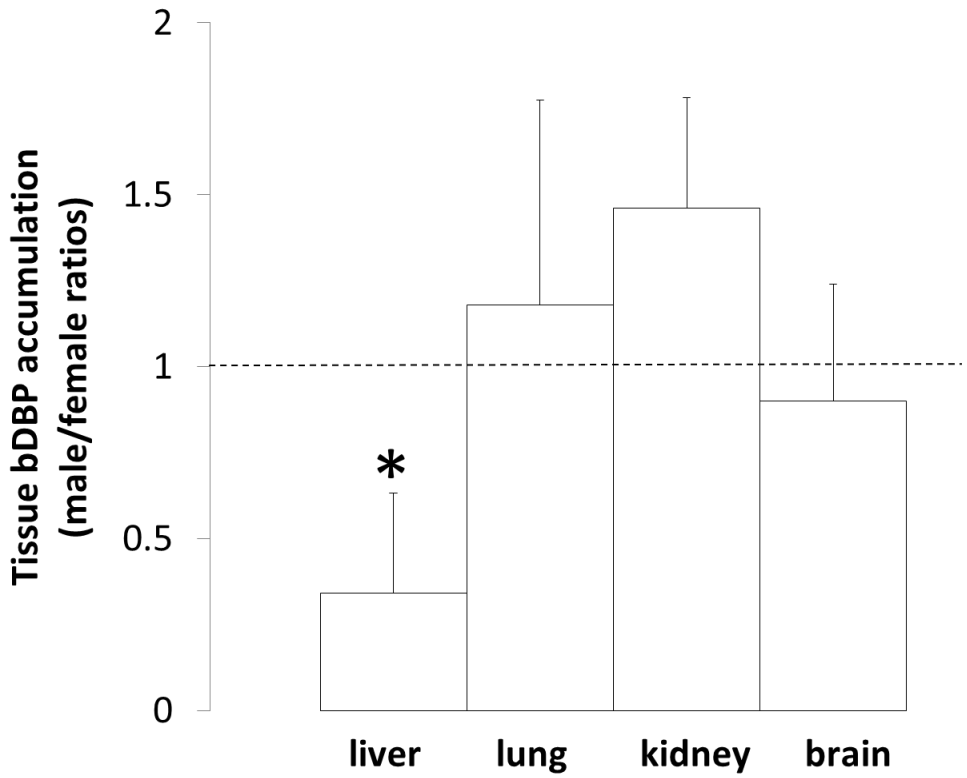

Figure 4-2 Comparison of b-DBP tissue accumulation to binding in terms of male/female ratios.

The accumulation to binding ratios of biotin-labelled DBP (b-DBP) in male and female mice are illustrated 15-20 min post injection. No significant differences are observed in DBP endocytic efficiency between male and female lung or brain tissue. When comparing male and female liver tissues, however, endocytic efficiency of DBP is significantly lower in the former (asterisk, $p<0.05$). ($n = 8$ for all tissues; replicates = 2 for each male and female)

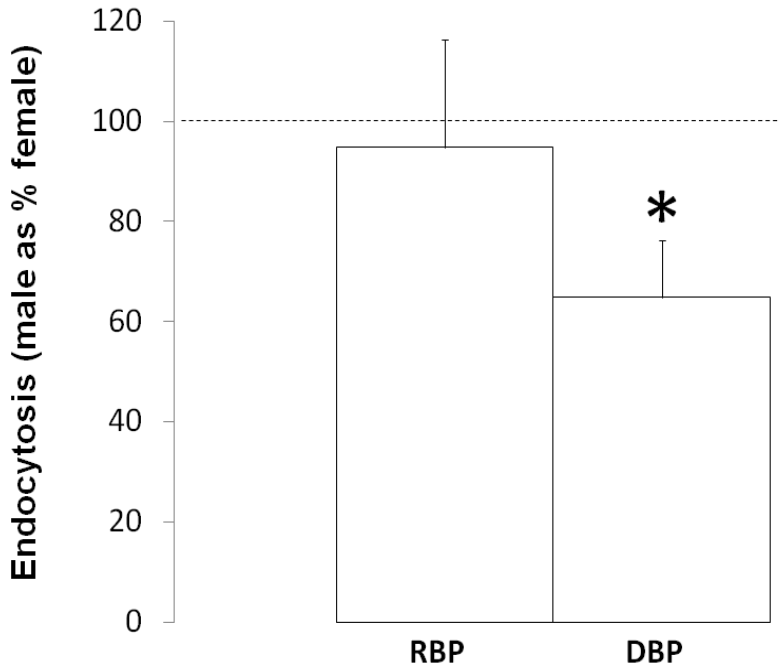

Figure 4-3 Comparison of two vitamin binding proteins, DBP and RBP, in terms of endocytic activity in male and female hepatocyte cells

The accumulation of biotin-labelled DBP is significantly lower in male hepatocytes compared to female hepatocytes. This effect, however, is not observed for biotin-labelled RBP in the same two cell types. (n = 6; replicates = 2 for both DBP and RBP)

In terms of aging, total DBP binding capacities were analyzed in membrane preparations from different tissues of young (~1.5 months) and old (~27 months) mice as described in Materials and Methods. Figure 4-4 shows b-DBP binding in liver, lung and kidney tissues. In all cases, there are decreases in binding capacity in old tissues relative to young. Borderline significance is achieved when comparing old and young liver tissues ($p=0.058$) and old and young kidney tissues ($p=0.061$). In addition, a significant difference is found between old kidney tissues and old liver and lung tissues (K, $p<0.01$) as well as between young kidney tissues and old liver and lung tissues (K, $p<0.01$) and between young liver and lung tissues (L, $p<0.05$). Although accumulation data was not obtained, the decrease in binding capacity may imply a potential decrease in b-DBP accumulation at early endocytic time points as has been observed with other nutrient carriers (Blanpied, Scott, & Ehlers, 2003; Chen, 2009; Le Couteur et al., 2008; Morgan & Moos, 2002). It has been well established that dietary requirements for vitamin D3 increase with age (Bailey et al., 2010; Llewellyn et al., 2010; Ross et al., 2011); and this relative lack of D3 may, in part, be due to inefficiency of DBP-mediated D3 uptake.

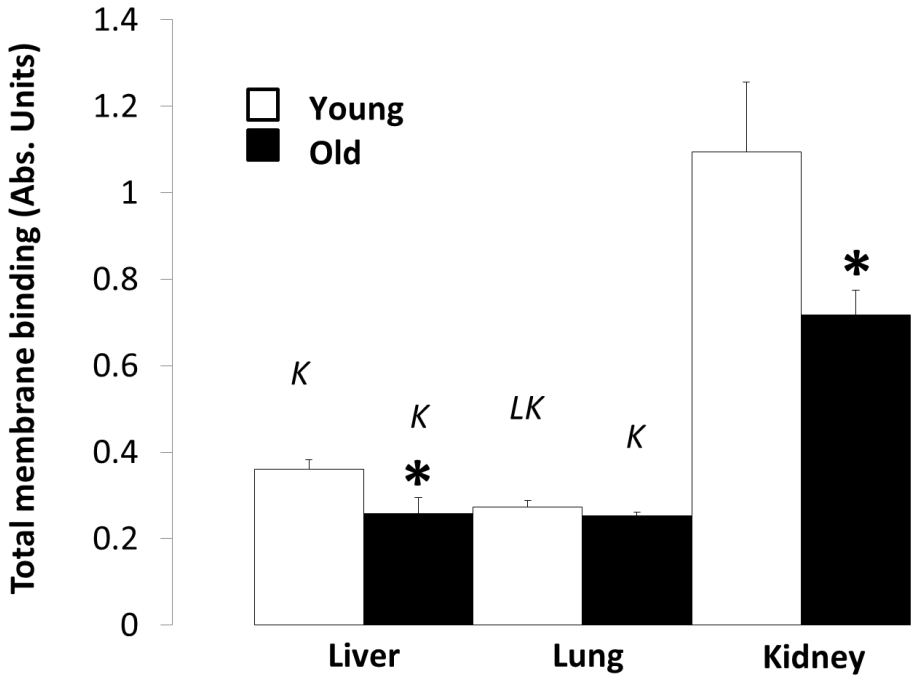

Figure 4-4 Effects of aging on tissue binding capacity of DBP.

The binding of biotinylated-DBP to membrane preparations from liver, lung and kidney tissues on young and old mice tissues is shown after an overnight incubation with the labelled nutrient carrier. Binding of b-DBP in old tissues appears to be decreased in all tissues when compared to binding in young tissue, however only borderline significance is obtained when comparing binding capacity in old and young liver (asterisk, $p<0.05$ for one tailed-t test only) and old and young kidney (asterisk, $p<0.05$ for one-tailed t-test only). A significant difference is found between old kidney tissues and old liver and lung tissues (K, $p<0.01$) as well as between young kidney tissues and old liver and lung tissues (K, $p<0.01$) and between young liver and lung tissues (L, $p<0.05$). ($n = 4$; replicates = 2 for all young and old tissues)

CHAPTER 5: MODULATION OF DBP TRANSPORT: SOME ADDITIONAL PHYSIOLOGICAL AND COMPARATIVE ASPECTS

5.1 Introduction

Modulation of nutrient transport has physiological and potential pathological consequences. Changes in such transport, for example may modulate the availability of biologically active nutrient metabolites that are critical for cell growth or differentiation. Below are some additional examples related to vitamin transport, including calciferols and DBP.

Biotin transport into cells has been related to chromatin remodelling and epigenetic effects (Zempleni, Gralla, Camporeale, & Hassan, 2009) with potential physiological and pathological consequences. Likewise, modulation of retinoid transport (vitamin A metabolites) may result in epigenetic changes (Corlazzoli, Rossetti, Bistulfi, Ren, & Sacchi, 2009), and may have consequences for the progression of Alzheimer's disease (Goodman & Pardee, 2003) and for

neurological functions (Brouillette & Quirion, 2008). Also in this context, a novel therapeutic strategy for liver cirrhosis has been reported that is based on RBP and vitamin A liposomes (Sato et al., 2008), and may involve RBP endocytosis.

In the case of calciferols and DBP, there is preliminary evidence that cell differentiation is associated with increased receptor-mediated endocytosis of DBP in mammary epithelial cells, and possibly in colonic and prostate epithelial cells (Ternes & Rowling, 2009). Rapid cell proliferation is often associated with de-differentiation (e.g., in the development and progression of cancers); in this context, studies for this thesis have examined DBP endocytosis in different states of cell growth and in the presence of growth modulators such as EGF. Different responses of A431 cells in terms of cell growth and differentiation have been reported (Barnes, 1982; Gill & Lazar, 1981; Kawamoto et al., 1983; Konger & Chan, 1993; Masui, Castro, & Mendelsohn, 1993). I have compared the effects of such potential growth-modulating parameters on DBP endocytosis.

5.2 Results and Discussion

Endocytic transport of carrier proteins that bind pro-differentiative factors such as retinoids and calciferols may be decreased in situations of increased cell proliferation. Alternatively, it may be argued that cells stimulated to proliferate may attempt to increase endocytosis of such carrier proteins for pro-differentiative factors as a feedback response to try to regain control of growth. These possibilities were tested on the epidermoid A431 cells. Cell growth parameters

were modulated both by using different cell growth densities and by treatment with epidermal growth factor (EGF); EGF can be a strong stimulator of epithelial cell growth.

The data (Figures 5-1, 5-2, and 5-3) suggest modulation of DBP endocytosis by cell growth parameters. The A431 cells were grown to low (25%), medium (50%) and high (90%) cell densities, incubated with b-DBP for 10 minutes as described in Materials and Methods, and the data was subsequently analysed by standardizing against the medium cell density control. In the case of different cell confluencies, the results shown in Figure 5-1 show no statistically significant difference in the uptake of DBP. Another nutrient carrier protein, transferrin (Tf), does exhibit a significant confluency-related modulation of endocytosis (b, $p<0.01$) and also shows significance with regard to increased endocytosis at high cell densities when compared to endocytosis of b-DBP at high cell densities (a; $p<0.01$). Figure 5-2 illustrates ratios of DBP to Tf accumulation: at low cell confluencies, b-DBP accumulation is greater than that of b-Tf; but at high confluencies, b-DBP accumulation is only about half that of its comparative control ($p<0.01$).

Binding studies between DBP and Tf were completed using a similar procedure to that above for endocytosis, with the exception that the labelled-nutrient carrier was incubated on ice to avoid protein uptake by cells. In Figure 5-3, a significant difference is observed between binding capacities of Tf and DBP at high cell confluencies, and for binding of Tf at low and high cell confluencies. No statistically significant difference is found between b-DBP

binding capacity at either cell density. In comparing the results of Figures 5-1 and 5-3, there appears to be a corresponding relation between binding and accumulation, i.e., both higher for DBP at low density and for Tf at high density.

To provide a more relevant context for the measurement of endocytic efficiency, accumulation was corrected for binding. Figure 5-4 shows that DBP endocytosis at high growth densities appears to be similarly efficient to Tf endocytosis at high confluencies. At low cell confluencies, however, there is a significant difference between the endocytic efficiency of DBP and Tf (b, in Figure), and between Tf at low and high cell confluencies (a, in Figure): cells at low confluency endocytose Tf more efficiently than DBP, and endocytose Tf more efficiently than at higher confluencies. This result suggests Tf-specific endocytic regulation based on cell growth density, and possibly on rate of cell growth.

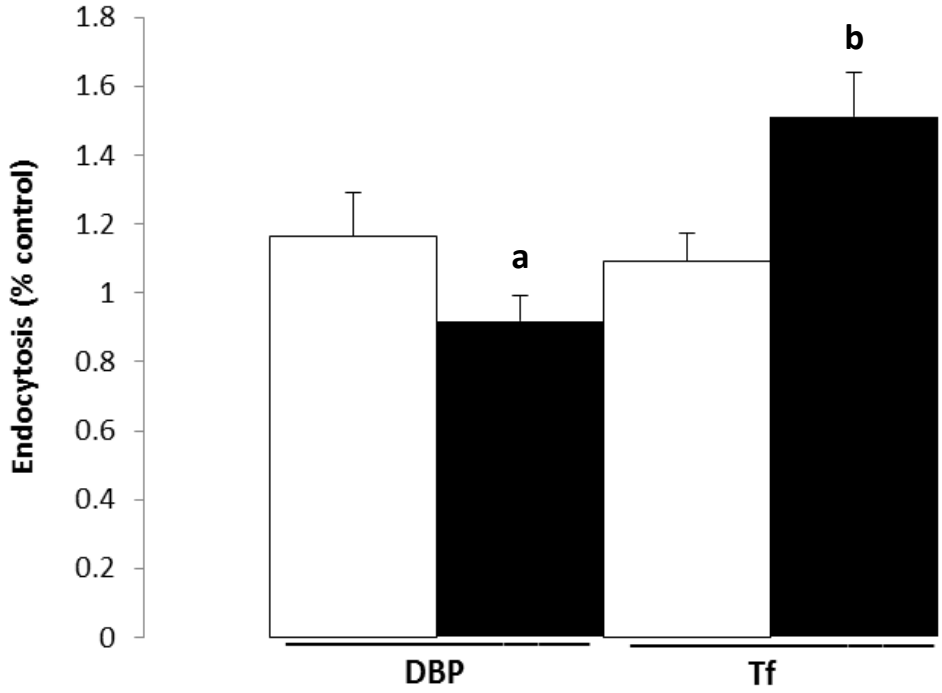

Figure 5-1 Relative effects of cell growth density on the accumulation of two nutrient carrier proteins, vitamin D-binding protein and transferrin.

Relative to an intermediate cell growth density (~50% confluency), cells at a low growth density (~25%, white bars) appear to be more efficient in terms of DBP endocytosis than those at high growth density (>90%, black bars); this difference, however, is not statistically significant (p=0.18). For Tf, a statistically significant difference between the two densities is observed, ***b****, p<0.01. A statistically significant difference between DBP and Tf endocytosis at high growth densities is observed,* ***a****, p<0.01. (n = 20; replicates = 9 for all cases)*

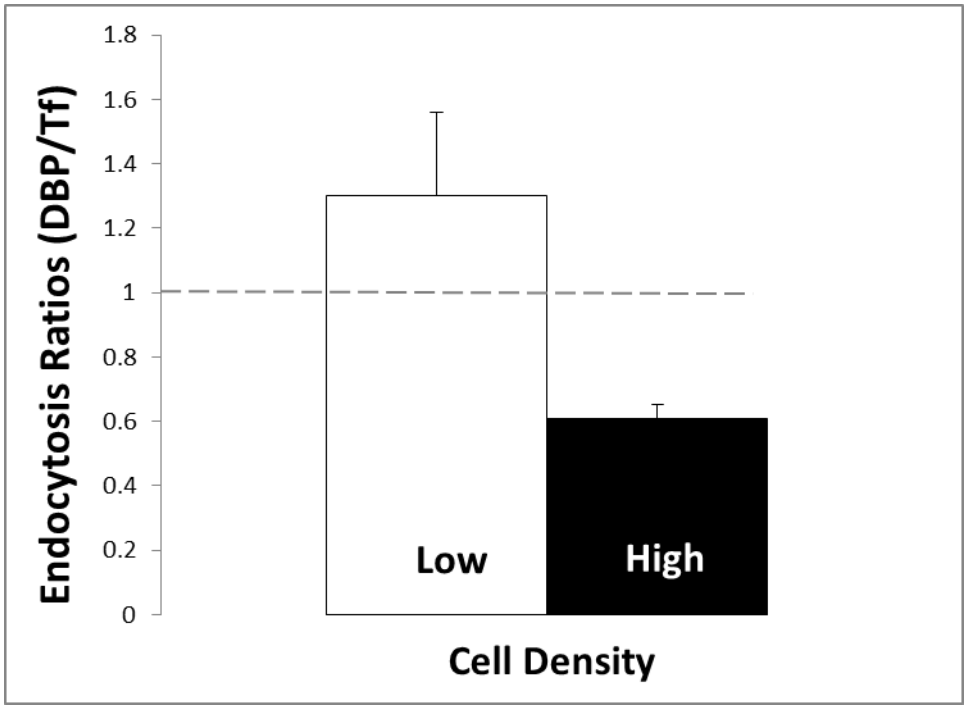

Figure 5-2 Comparison of the accumulation efficiency of DBP and Tf.

The relative accumulation of DBP to Tf is shown after a 10 min endocytosis period. The accumulation of DBP in A431 cells at low growth (25%, white bars) densities is similar to that of Tf; but at high growth (>90%, black bars) densities DBP accumulation is only about half as efficient as Tf accumulation ($p<0.01$). ($n = 20$; replicates = 9 for both DBP and Tf).

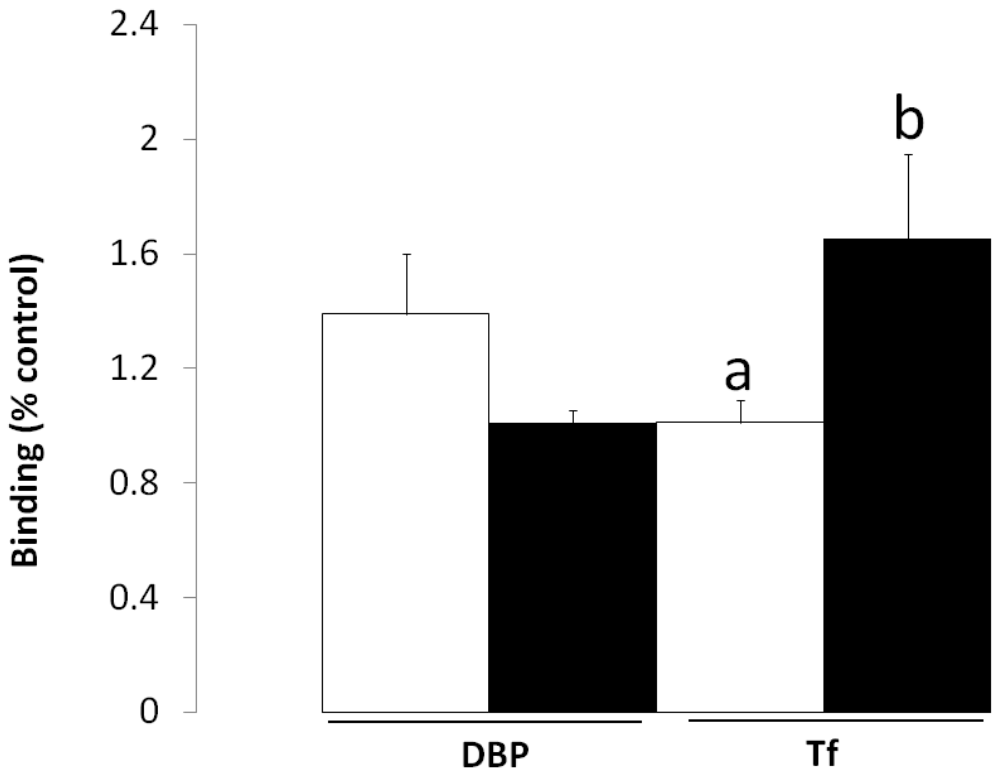

Figure 5-3 Relative effects of cell growth density on the membrane binding of two nutrient carrier proteins, vitamin D-binding protein and transferrin.

Relative to an intermediate cell growth density (~50% confluency), cells at a low growth density (~25%, white bars) appear to have a similar (difference is not statistically significant) binding capacity for DBP when compared to a high growth density (>90%, black bars). However, low confluency cells seem to have a lower binding capacity for Tf. A significant difference is observed between binding of Tf between low and high cell confluencies (a) and for binding capacities of Tf and DBP at high cell confluencies (b). (n = 6; replicates = 3 for all cases)

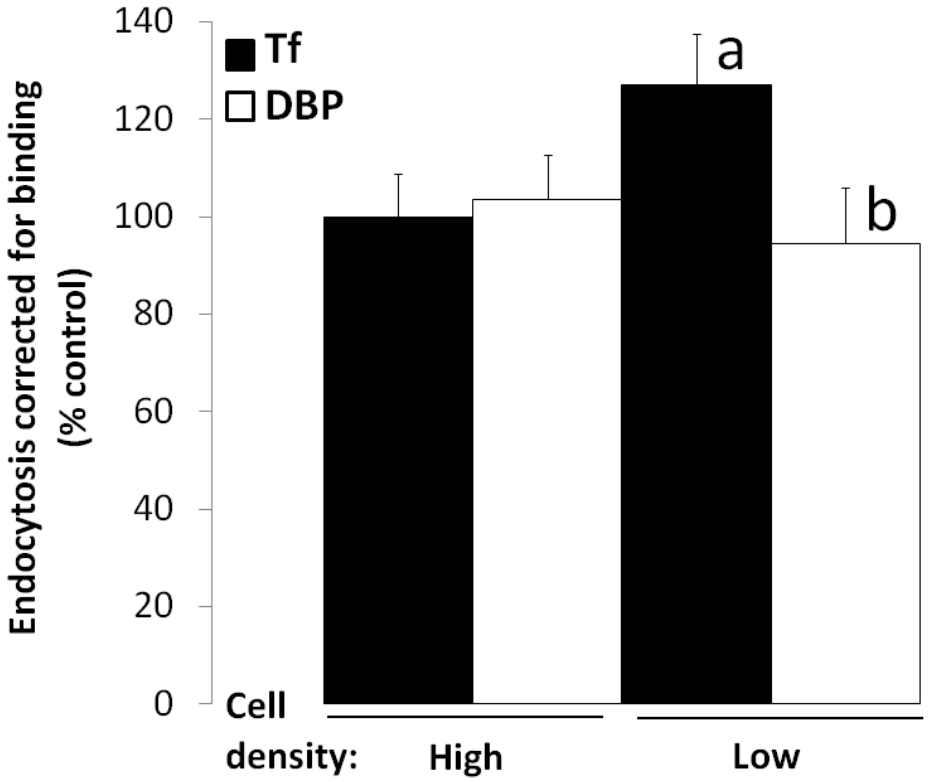

Figure 5-4 Relative effects of cell growth density on the endocytosis of two nutrient carrier proteins, DBP and Tf.

Relative to an intermediate cell growth density (~50% confluency), cells at a low growth density (~25%) endocytose significantly (b; $p<0.05$) more Tf than DBP. In addition, significantly (a; $p<0.05$) more Tf is endocytosed at lower confluencies than at higher confluencies (~90%). Both nutrient carriers seem to be similarly efficient at higher cell growth densities. (n=20; replicates = 9 for all cases)

In terms of the effects on DBP endocytosis caused by EGF treatment of cells (A431), there does seem to be modulation of DBP transport by this growth factor. Cells grown to 50% and 90% confluencies were incubated with 5 nM EGF for 30 min prior to DBP endocytosis as described in Materials and Methods. When looking solely at accumulation (Figure 5-5), the presence of this growth factor does not appear to provide a statistically significant stimulation of DBP transport at either low or high cell growth densities.

DBP binding studies under treatment of EGF were also performed. DBP binding studies were completed using a similar procedure to that for endocytosis above, with the exception that after EGF treatment, the labelled-nutrient carrier was incubated on ice to avoid protein uptake. Figure 5-6 illustrates that DBP binding capacity is similar with or without EGF treatment at the two cell growth densities.

When DBP endocytosis is standardized for DBP binding (Figure 5-7), EGF treatment at different cell growth densities does seem to affect the efficiency of DBP transport. A significantly higher standardized uptake of DBP is found with EGF treatment at lower cell growth densities compared to higher densities. These data suggest that cells at different growth densities react differently to EGF treatment. At low growth densities EGF treatment may induce A431 cells to increase their uptake of DBP (and other prodifferentiative factors) as a counter-regulatory mechanism to try to maintain control of cell growth in the presence of the proliferative factor. In this context, A431 cells have been found to

increase caveolar endocytosis activity in response to EGF (Orlichenko et al., 2009) and references therein).

In order to test if 5 nM of EGF enhances metabolic activity within this 30 min treatment period, MTT [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide] tests with and without EGF treatment were performed. Enhanced metabolic activity typically coincides with proliferative potential (Bauer, Hatzivassiliou, Zhao, Andreadis, & Thompson, 2005; Fritz & Fajas, 2010; Vander Heiden, Cantley, & Thompson, 2009) (L. Yang, Liang, Yao, Chen, & Hou, 2005). Figure 5-8 illustrates that cells at high (90%) cell densities show a small, but statistically significant, acute increase in their metabolic activity with EGF treatment ($p=0.05$). If one uses metabolic activity as an indicator of proliferative potential, these results suggest that acute treatment with EGF may activate some proliferative pathways in A431 cells, especially at higher confluencies. The relative decrease in DBP endocytosis, and hence the decrease in endocytosis of its pro-differentiative ligands, observed in cells at high confluency (Figure 5-7), correlates with the increase in metabolic activity seen in response to EGF treatment (Figure 5-8). Thus, I suggest that at relatively low growth densities (e.g., 50%), EGF stimulates endocytosis, and the correlation with relatively lower MTT conversion may be a result of the increased uptake of pro-differentiative factors such as DBP (vitamin D) that help control the extent of increases in cellular metabolic activity or growth potential; such an EGF-dependent stimulation in endocytosis may not occur at high growth densities (e.g., 90%).

Acute metabolic responses to 5 nM of EGF treatment seem to differ from those of longer-term treatments. Data from A431 cells incubated with EGF for 48 h at both low and high cell densities show decreases in MTT conversion relative to untreated cells (Figure 5-9; statistically significant when using a one-tailed t-test; low cell confluency, $p=0.05$ and high confluency, $p=0.03$). One possibility is that short term treatment with EGF may act as a growth modulator that increases proliferation, but that prolonged exposure allows for a variety of sequential cellular changes that may result in inhibition of proliferation, and potentially apoptosis. Some studies have found that nanomolar concentrations of EGF can inhibit proliferation in A431 cells (Barnes, 1982; Gill & Lazar, 1981; Masui et al., 1993); but the potential contribution of apopotosis to these results is not known. Another possibility is that over the longer term, i.e. 48 h, the EGF treatment decreases the number of mitochondria—a major location of MTT conversion—especially in the higher growth density conditions.

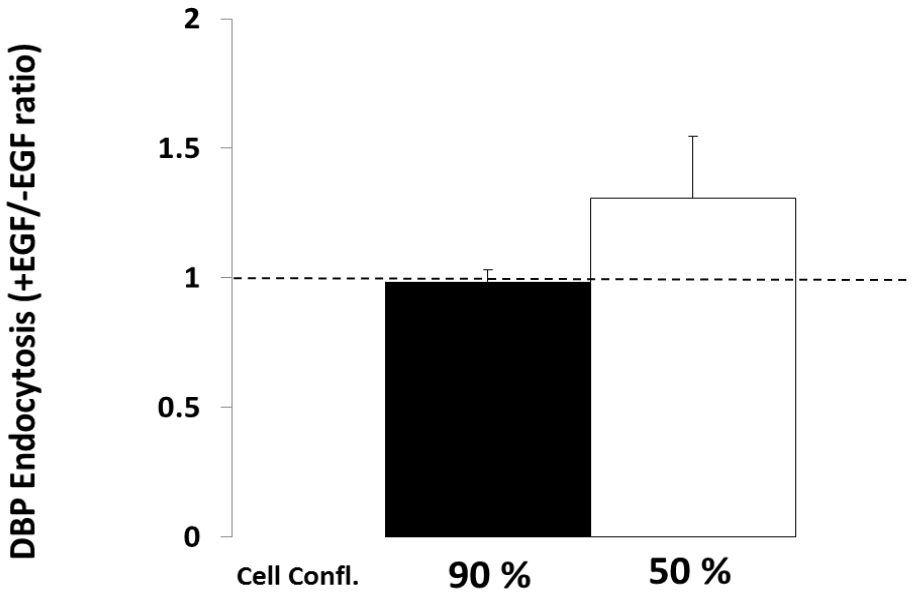

Figure 5-5 Effects of epidermal growth factor on cellular DBP accumulation at two different cell growth confluencies.

A431 cells in serum-free medium, at 50% (white bar) and 90% (black bar) plate confluency, were incubated with 5 nM EGF for 30 min prior to assessing endocytosis by incubating the cells with DBP for 10 min at 37°C. The EGF treatment does not result in a statistically significant difference between the two cell growth densities. (n = 6; replicates = 3 for both densities)

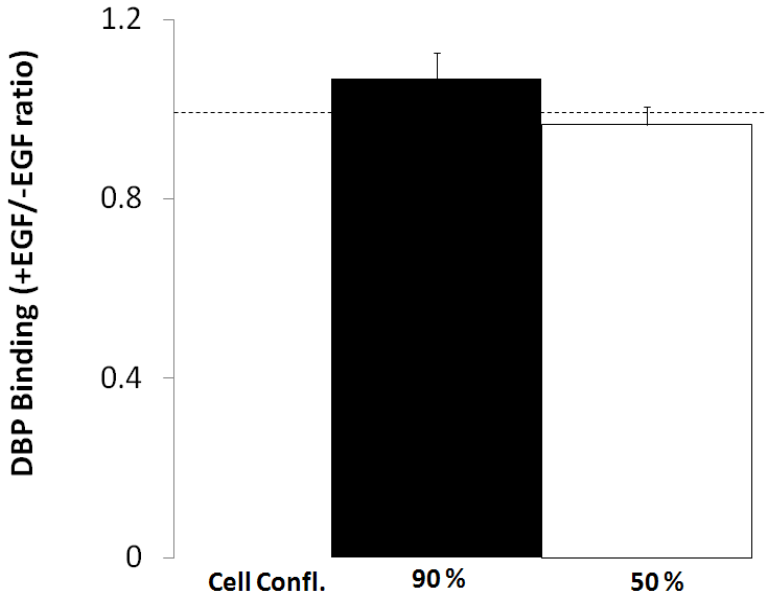

Figure 5-6 Effects of epidermal growth factor on DBP binding at two different cell growth densities.

A431 cells in serum-free medium, at 50% (white bar) and 90% (black bar) plate confluency, were incubated with 5 nM EGF for 30 min prior to assessing DBP binding capacity by incubating the cells with DBP for 10 min on ice. EGF treatment does not appear to significantly affect DBP binding capacity at either cell density. (n = 6; replicates = 3 for both densities)

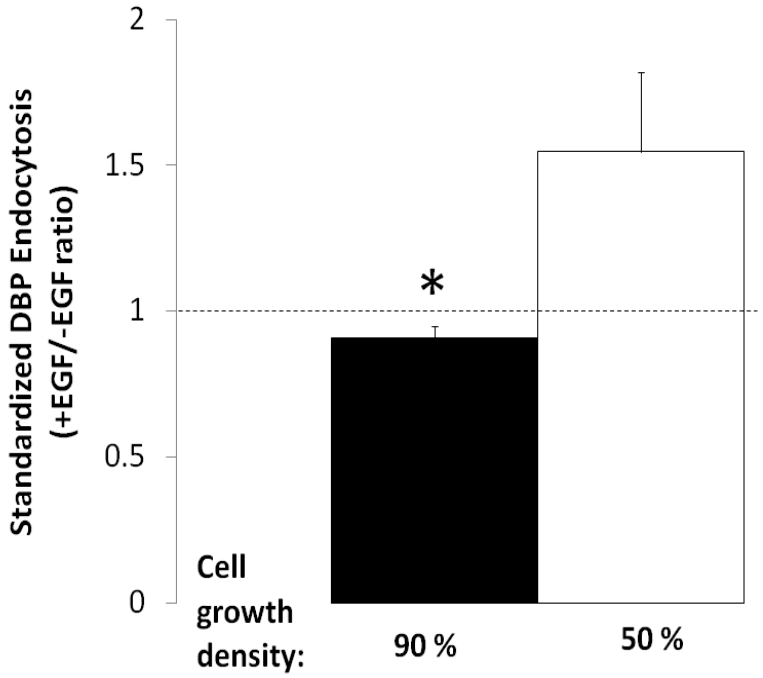

Figure 5-7 Effects of epidermal growth factor on DBP endocytosis corrected for binding.

A431 cells at a 50% (white bar) cell growth density exposed to 5nM of EGF for 30 min endocytose significantly more DBP than at a 90% (black bar) cell growth density ($p<0.05$). ($n=6$; replicates = 3 for both densities)

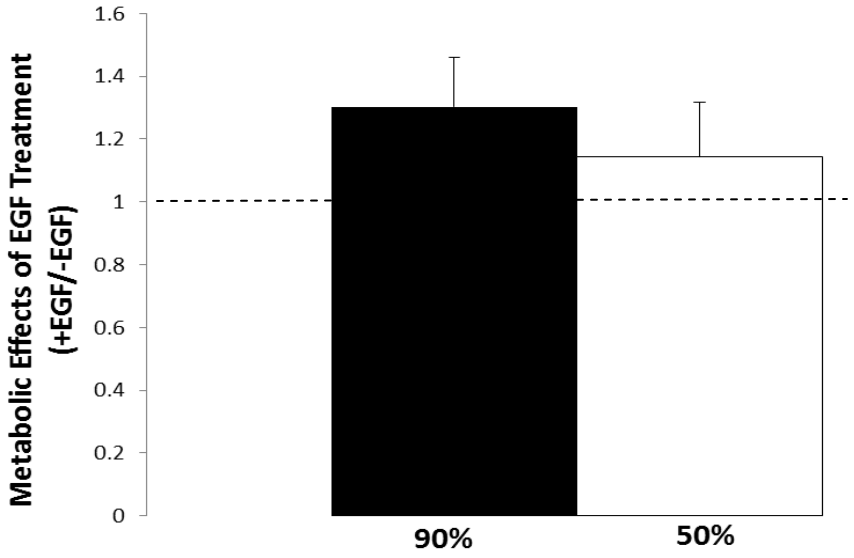

Figure 5-8 Metabolic effects of 30 minute EGF treatment at two different cell growth densities

Cells at both low (50%) and high (90%) cell growth density underwent a 30 min treatment with EGF. Cells at high growth confluency show a small, but statistically significant increase in metabolic activity in comparison to the control (p=0.05). (90% (and 90% controls) n = 14; 50% (and 50% CONTROLS) n = 12; replicates = 2 for both cases).

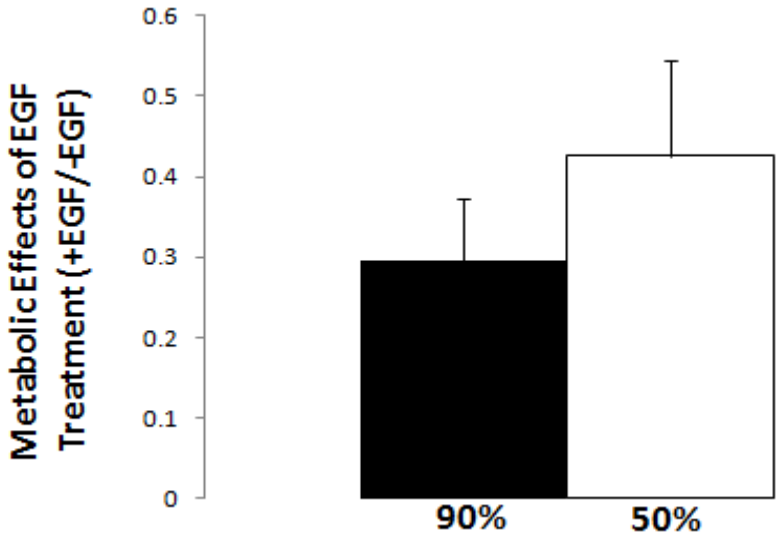

Figure 5-9 Metabolic effects of 48 hour EGF treatment at two different cell growth densities

Cells at 50% and 90% cell growth density underwent a 48 h treatment with EGF. Cells at both confluencies show decreases in metabolic activity in comparison to the control (statistically significant when using a one-tailed t-test; low cell confluency, p=0.05 and high confluency, p=0.03). The difference between the two growth densities is not statistically significant (p=0.4). (90% n = 10, 50% n = 8, (50 and 90%) controls n = 5; replicates = 2)

In the context of gender-related modulators of DBP endocytosis discussed in Chapter 3, I analyzed the effects of estradiol treatment on DBP endocytosis in hepatocytes. Estradiol is a sex hormone and is the predominant estrogen found in premenopausal females (small quantities can be present in males as a testosterone metabolite). As shown previously in Figure 3-4, this sex steroid does not stimulate DBP endocytosis in male hepatocytes. As shown now in Figure 5-10, however, female hepatocytes do exhibit a significantly strong estradiol-dependent increase in DBP endocytosis. There has been previous evidence of estradiol related increases in endocytic uptake of various factors, specifically in females (Handley, Arbeeny, & Chien, 1983; Morris, Potter, & Gaza-Bulseco, 1988; L. Yang et al., 2005). I suggest that estradiol increases the efficiency of DBP RME in female cells. Perhaps this steroid hormone activates one or more components of the endocytic machinery that are more abundant in female cells, or activates a female cell-specific signalling pathway that increases endocytic activity.

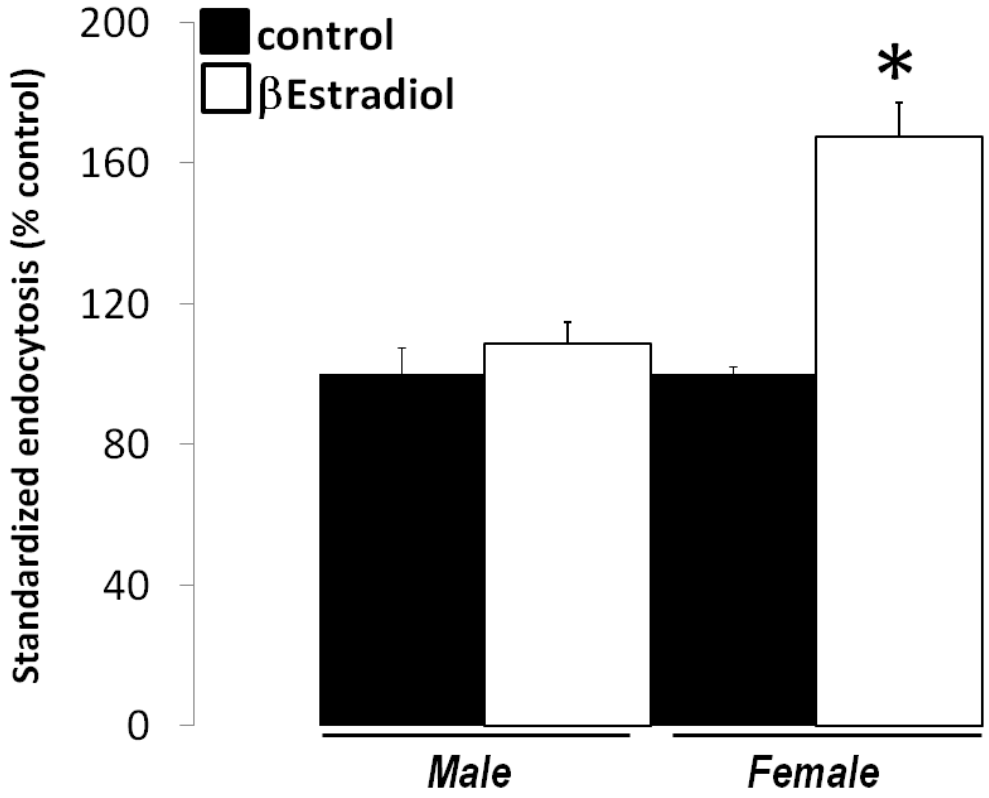

Figure 5-10 Effects of estradiol on DBP endocytosis in male and female hepatocytes.

Male and female hepatocytes were treated with estradiol for 30 min prior to analysis of DBP endocytosis. Treatment with estradiol significantly increases DBP endocytosis in female hepatic cells when compared to the control (no treatment). No significant differences in DBP internalization are found in male hepatocytes with or without estradiol exposure. (n = 4; replicates = 2 for both male and female)

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The following list encompasses the major conclusions found from the experiments completed as part of this M.Sc thesis project:

(i) Functional receptors exist on the surface of human A431 cells and liver, lung, kidney, adipose and brain tissues in mice.

(ii) DBP-binding activity is higher in both intact A431 cells and cell membrane fractions when compared to Tf.

(iii) Male liver cells have a decreased DBP endocytic capacity relative to female liver cells, perhaps because female hepatocytes have a higher level or activity of one of the many factors that can influence endocytic efficiency.

(iv) Filipin, a known inhibitor of the caveolin-mediated endocytic pathway, significantly decreases the amount of DBP endocytosed in hepatocytes, which suggests a caveolar endocytic pathway is employed by these cells.

(v) Endocytic transport of DBP and Tf may be influenced by cell growth status.

(vi) A431 cells at low cell density (50%) increase their transport of DBP (and its pro-differentiative ligands) with acute (30 min) EGF treatment, which may result as a counter-regulatory response to the presence of this growth factor (assuming EGF has a proliferative effect on cells at low density). Acute EGF treatment significantly decreases DBP transport at high cell densities (90%), in comparison.

(vii) A431 cells grown at high cell densities (90%) show metabolic stimulation with acute (30 min) EGF treatment. Using metabolic stimulation as an indicator of proliferation potential, this stimulation in metabolism and likely proliferation, may be due to the decreased uptake of DBP (and its pro-differentiative ligands) that occurs with this short term EGF treatment.

(viii) Long term EGF treatment (48 h) decreases metabolic activity in cells at both low and high density (50% and 90%, respectively). This may be due to cellular changes that occur with prolonged EGF exposure that may lead to inhibition of proliferation and eventually apoptosis.

(ix) Liver and kidney tissues of old mice have decreased binding activity when compared to tissues of young mice, which is consistent with the age effects of other nutrient carriers such as RBP and Tf.

(x) Estradiol significantly increases DBP endocytosis in female hepatocyte cells (compared to control and to male hepatocyte cells), which suggests

that this estrogen may stimulate (DBP) RME in female cells, as has been seen with other nutrient carriers.

6.2 Future directions

To expound on the research completed above, the following are future directions that would be most pertinent to explore:

- Characterize the potential DBP-specific receptor in terms of its (i) identification (amino acid sequence), (ii) size, and (iii) presence in various cells and tissues.
- Further characterize DBP endocytosis in terms of (i) other putative endocytic pathways of DBP with the use of pathway inhibitors, and (ii) whether its endocytosis is ligand-dependent.
- Further establish the (proliferative) effects of EGF on A431 cells by completing cell-counting assays and assays to assess the extent of apoptosis after prolonged exposure.
- Further establish age-related effects on DBP accumulation and endocytic efficiency in tissues.
- Given its effects on increasing DBP uptake in females, explore estradiol as a potential therapy for the treatment of osteoporosis in post-menopausal females.

APPENDIX: NOVEL MURINE HEPATOCYTE PREPARATION METHOD

Isolated animal liver cells have been used in a wide range of biochemical experiments from hepatic function to basic process of cell biology. Typically, the isolation process involves treatment of the livers with collagenase or other proteases. These enzymes, however, can digest cell surface proteins and change membrane-related cell functions. Such cells cannot be used in assays that test or rely upon some of these functions, e.g., receptor-mediated endocytosis (RME). Non enzymatic methods have been reported, and most of these are based on the use of chelators such as EDTA (Kravchenko et al., 2002 and references therein). We have developed a simplified EDTA-sucrose-based method that does not require motorized mechanical disruption. The method was applied for the preparation of mouse liver cells, and yields functional cells, ready to be used without the need for typical multi-day culture.

The following is the liver isolation procedure that we used. After perfusion of the whole circulatory system with 30 ml of physiological saline, the gall bladder is extracted, and the liver is removed and perfused with 2 ml of PBS followed by 2 ml of EDTA-sucrose (ES) solution (250 mM Sucrose and 4 mM EDTA; Kravchenko et al., 2002). The latter perfusions of the isolated liver can be performed while holding it with forceps and injecting the solution slowly at multiple sites in the different lobes; the perfusate drips out of the liver and is discarded.

In a Petri dish containing 2 ml of ES, the liver is torn into the smallest possible pieces using a pulling motion with 2 forceps over a 3 min period. The pieces are left to incubate in the ES solution for a further 5 min. The contents of the Petri dish are then gently passed 5 times through a 5-ml syringe (no needle), 5 times through a 1-ml pipette tip with 8 mm of tip cut off, 5 times through a 1-ml pipette tip with 4 mm of tip cut off, and transferred into collection tubes (1.5 ml Eppendorf). This initial cell suspension is then briefly centrifuged at low g-force (10 s, 50 *x g*) to remove tissue debris. The supernatant is transferred to new tubes, gently passed once through an 18 gauge needle, 3 times through a 23 gauge needle, subsequently centrifuged at 50 x g for 3.5 min to pellet the cells.

The cell pellets are gently resuspended in 1 ml of complete cell culture medium (DMEM-FBS) and re-centrifuged, 100 x g for 3 min. The supernatant is discarded and the pellet is gently resuspended in about 2 volumes of PBS-BSA-SFM (1 volume [phosphate-buffered saline, pH 7.4, containing 1% w/v bovine serum albumin] mixed with 1 volume [serum-free cell culture medium, DMEM]), and kept at room temperature for at least 1 h (less than 4 h) before being used for experiments. Resuspended pellets from multiple tubes can be combined into 1 or 2 tubes. The total volume of the cell suspension can be measured at this point, and a small sample of cells removed for trypan blue exclusion (viability) test and cell counting. If desired, some of the cells can also be frozen (in typical high serum-DMSO solutions) and stored in liquid nitrogen for future use.

The simple, rapid EDTA-based method presented here typically yields 15-30 million cells from an adult mouse liver. Cell viability estimated by trypan blue dye

exclusion is very high, typically above 98%. Figure A-1 shows a light microscope picture of typical isolated cells. We employed these cells for endocytic transport assays and found them to be highly active. Moreover, cells prepared by similar EDTA-based methods have previously been reported to have the same or higher activity in terms of energy metabolism (Kravchenko et al., 2002 and references therein).

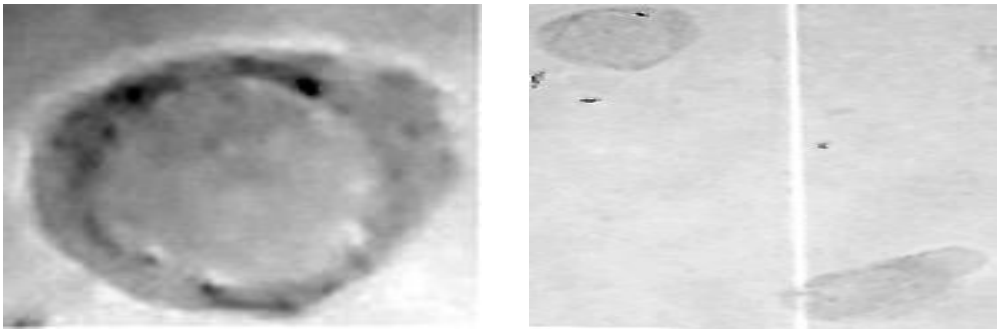

Figure A-1 Murine hepatocytes prepared by the simplified EDTA-sucrose method.

Examples of low (right) and high (left) power magnification photographs are shown of hepatocytes prepared by the novel EDTA-sucrose method outlined in this Appendix.

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