PHYSIOLOGICAL MECHANISMS OF NUTRIENT TRANSPORT: VITAMIN A AND RETINOL-BINDING PROTEIN

by

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ABSTRACT

Biologically active metabolites of retinol (vitamin A) affect growth, differentiation, and survival of cells. Retinol-binding protein (RBP) is a specific circulatory transport protein for retinol and participates its delivery to cells. Based on evidence for receptor-mediated endocytosis of RBP, experiments were carried out to further characterize (a) the RBP receptor and (b) the fate of endocytosed RBP in a human epithelial cell line. Additional experiments were performed with mice to examine (c) possible age-related changes in tissue accumulation of injected RBP. The results provide the first size characterization of an RBP receptor in these cells, a 157 kDa species, and the first evidence for recycling of endocytosed RBP. Age-related decreases in transport were suggested by a 23-27% decrease in the accumulation of exogenous RBP in old vs. young tissues (early post-injection times). Additional work on related transport mechanisms was carried out for RBP and the iron carrier transferrin.

Keywords:
Retinol-binding protein; Aging; Receptor-mediated endocytosis; Endocytic trafficking; Vitamin A; Transferrin; Phytochemicals

Subject Terms:
Nutrition and physiology; Cell biology and biochemistry

EXECUTIVE SUMMARY

Biologically active metabolites of vitamin A (retinol) participate in the control of various physiological functions through their effects on cell growth, differentiation, and death. Vitamin A is transported through the blood to different body tissues as a complex with retinol-binding protein (RBP), a member of the lipocalin protein family. The molecular mechanisms by which retinol is obtained by cells from the retinol-RBP complex are poorly understood; a major objective of this thesis project was to gain a more systematic and detailed understanding of these mechanisms. Experiments were carried out with the following specific goals in an attempt to obtain novel information and applications related to such molecular and cellular transport:

- to further characterize the plasma membrane RBP-receptor(s) in a human epithelial cell line;

- to determine if RBP can recycle out of the cell after endocytosis in a human epithelial cell line;

- to compare the accumulation of labelled, exogenous RBP in tissues of old and young mice;

- to compare some of the RBP transport parameters with those of the iron-carrier protein transferrin (well-established control) and other nutrient transport systems;

The results of the experiments (a) provide the first size characterization of an RBP receptor in the epithelial cell line (A431): a 157 kDa species was chemically cross-linked to labelled RBP. (b) The first evidence, in any cell type, was also obtained for recycling of endocytosed RBP; loss of internal labelled RBP was observed as well as appearance of such RBP in the growth medium. (c) Decreased accumulation of injected, labelled RBP was observed in liver and kidney tissues of old vs. young mice (30 min. post injection), without any decrease in total tissue membrane RBP-binding capacity. These accumulation results may be indicative of age-related decreases in transport; a ratio of such RBP accumulation levels to total RBP binding capacity suggests a decrease in endocytic efficiency of about 25 percent in all tissues examined, a value comparable to those suggested by other aging studies (25-51% decrease) involving other cell types and ligands such as transferrin.

Overall, this thesis project provides novel information related to the physiology of RBP transport, and comparative information that relates RBP

transport to that of other nutrient carriers such as transferrin. In addition, an assay was developed that may be used as part of a strategy for screening of potential modulators of endocytic transport, e.g., in screening pharmaceuticals and dietary phytochemicals to identify stimulators or inhibitors of clathrin-mediated endocytosis.

DEDICATION

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

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I would like to express my gratitude to the people who contributed to the completion of the research and this degree.

Foremost I would like to thank my senior supervisor, Dr. Amandio Vieira, for his invaluable guidance and mentorship throughout my academic research. I really appreciate his unlimited patience, scientific inspiration, strong supports, enormous help and consideration in every single detail.

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GLOSSARY

atRA	All-trans retinoic acid
A431	Human epithelial (keratinocyte) cell line
b-X	Biotinylated-X (X = RBP, Tf, or other ligand)
BNHS	Biotin N-hydroxysuccinimide ester
BSA	Bovine serum albumin
CME	Clathrin-mediated endocytosis
COS-1	CV-1 simian cells transformed by origin-defective mutant of SV40 which codes for wild-type T antigen
CRABP	Cellular retinoic acid-binding protein
CRBP	Cellular retinol-binding protein
CV-1	Normal African green monkey kidney fibroblast cells
DMEM	Dulbecco's modified eagle medium
EB	Extraction buffer
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)
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase (plant origin)
Ig	Immunoglobulin (different types, e.g. IgG immunoglobulin G)
KSHM	100mM Potassium acetate, 85mM sucrose, 20mM Hepes, 1mM magnesium acetate, pH 7.4
LCAT	Lecithin-retinol-acyl transferase
LSEC	Liver sinusoidal endothelial cells
M	Molar (moles/litre); mM = milliM, etc.
OPD	*Ortho*-phenylene diamine
PBS	Phosphate-buffered saline
PI	Post-injection of animals or (p.i.) post-incubation of cells
RA	Retinoic acid
RAR	Retinoic acid receptor (nuclear)
RBP	Retinol-binding protein
RME	Receptor-mediated endocytosis
RNAi	RNA interference
RPE	Retinal pigment epithelial
RXR	Retinoid X receptor (nuclear)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFM	Serum free medium
SICM	Semi-intact cells and membranes

STRA6	Stimulated by retinoic acid gene 6 (retinoid transporter)
SV40	Simian vacuolating virus 40 or Simian virus 40
TBS	Tris-buffered saline
TBST	Tris-buffered saline with 0.1% Tween 20
Tf	Transferrin
TTR	Transthyretin (previous name: pre-albumin)
WiDr	A human colon carcinoma cell line

CHAPTER 1: INTRODUCTION AND MAIN OBJECTIVES

1.1 Overview of project and main objectives

Biologically active metabolites of vitamin A (retinol) have a wide range of physiological functions based on their ability to affect cell growth, differentiation, and death. Vitamin A is obtained from the diet, stored primarily in the liver, and transported through the blood to different body tissues as a complex with retinol-binding protein (RBP). The molecular mechanisms by which retinol is absorbed by cells from the retinol-RBP complex are poorly understood.

The *main objectives* of this MSc thesis project are to gain a more systematic and detailed understanding of (**a**) RBP receptors, (**b**) endocytic transport of RBP, and (**c**) potential effects of aging on RBP tissue accumulation and cell transport, i.e., aging as a potential modulator of

transport. In addition, (**d**) there is also a secondary goal of studying other modulators of nutrient transport, for RBP and other nutrient carriers such as transferrin (Tf), and trying to identify possible pharmacological modulators of endocytic transport.

More specifically, experiments have been carried out with the following goals, each represents an attempt to obtain novel information and applications related to this field:

- to characterize the plasma membrane RBP-receptor(s) in a human epithelial cell line;

- to determine if RBP can recycle out of the cell after endocytosis;

- to compare the extent of labelled-RBP accumulation in tissues of old and young mice;

- to compare some of the RBP endocytic transport parameters with those of Tf (well-established control) and other nutrient transport systems;

- to develop an assay for testing possible modulators of endocytic transport and test some phytochemicals and other dietary compounds currently studied in the laboratory.

The main experiments related to the physiology of RBP transport are shown schematically in Figure 1-1. As shown in the figure, these experiments are carried out using both isolated cells and animals, *in vivo*.

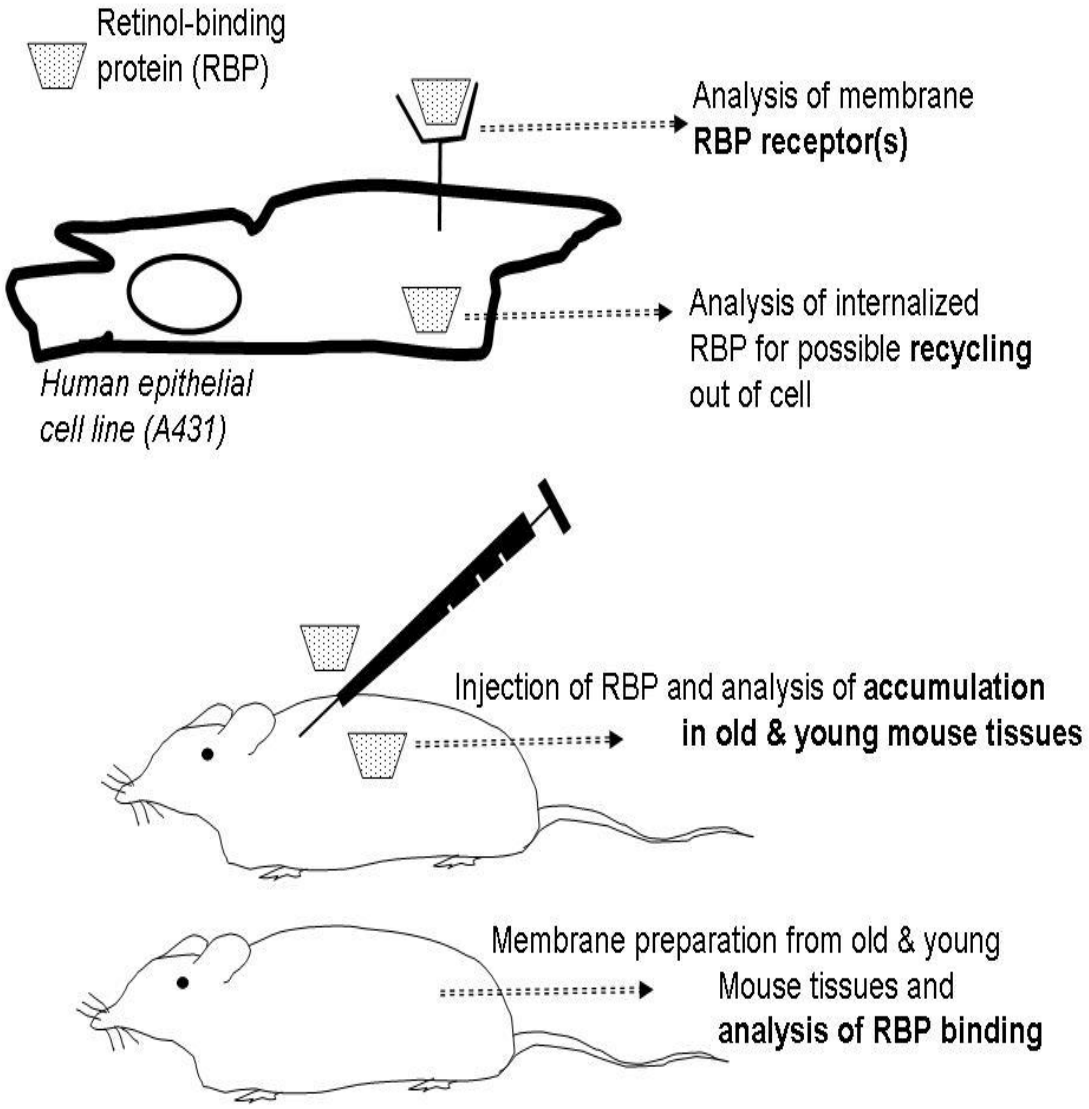

Figure 1-1 Schematic summary of major experiments related to the physiology of RBP transport that were carried out as part of this thesis work.

The **upper panel** shows the experiments on cultured cells (A431). Analysis of membranes was performed and led to the identification of an RBP receptor through chemical cross-linking procedures. Analysis of the fate of internalized RBP was also performed and provided evidence for recycling of RBP back out of the cell. The **lower panel** shows the experiments performed on animals (mice). Labelled RBP was injected into young and old animals, and tissue accumulation of the exogenous RBP was analyzed in different tissues (lung, liver, kidney); these experiments provided evidence for decreased accumulation in tissues from old animals. Non-injected animals (lower mouse figure) were also used as sources of tissue material; such tissue material was used for crude membrane preparations and analysis of total RBP binding to the membranes. Unlike the case with tissue accumulation, binding of RBP to the membranes prepared from young and old tissues did not decrease.

1.2 Background information

1.2.1 Retinoids

Vitamin A (Retinol) is the precursor of biologically active retinoids such as retinoic acids (RAs) that have been studied in many physiological and pathological contexts such as effects on gene expression and suppression of carcinogenesis in many cell types, especially epithelial cells (Stellmach et al., 1991; Lippman et al., 1995; Vieira, 1998; see structures of main retinoids in Figure 1-2). There are hundreds of retinoids, natural and synthetic derivatives of vitamin A (Fields et al., 2007). Biologically active retinoids participate in many physiological functions such as those of the immune system, reproduction and development, vision, maintenance of epithelial and other tissues (De Luca, 1991; Vieira, 1998; Niederreither and Dollé, 2008). Vitamin A deficiency can lead to blindness, bone deformities, testicular atrophy, embryonic death, immunodeficiency, increased risk of some cancers (Shils, 2006; Lotan, 1996; Fields et al., 2007). Vitamin A is usually absorbed as a provitamin from dietary sources such as β-carotene and other provitamin A carotenoids (mainly plant foods), or as preformed retinol esterified with a fatty acid (foods from animal products) (Fields et al., 2007; Vieira, 1998).

The major dietary sources of vitamin A are the provitamin A carotenoids, especially β-carotene, from green-leaf vegetables, carrots, sweet potatoes (Fields, et al., 2007). β-carotene itself can be cleaved by an oxygenase enzyme present in the intestinal epithelium to form retinal (the aldehyde form, cf. Figure 1-2); and the retinal, whose function is mainly related to vision, can either be oxidized by retinaldehyde dehydrogenases to all-trans RA (atRA) or reduced by retinaldehyde reductases to retinol (Fields, et al., 2007).

Retinol is the main form of vitamin A transported in the body's circulation. There are stores of retinol in the body, mainly in the liver. For storage, retinol is esterified with a fatty acid and stored as retinyl esters (Vieira 1998). Retinyl esters can be obtained from the diet as part of foods, e.g. meat products, especially organ meats such as liver; retinyl esters are often used to fortify foods such as milk, butter, margarines. Retinyl esters from foods can be hydrolyzed into retinol in the small intestine; retinol is then absorbed by intestinal mucosa cells and combined with a cellular retinol-binding protein (CRBP) inside the cell (Fields et al., 2007). Retinol can then be re-esterified by lecithin-retinol-acyl transferase (LCAT) in the intestinal mucosa cells and delivered by chylomicrons to some body tissues, mainly to the liver for storage (Fields, et al., 2007). When the body needs vitamin A, retinyl esters in the liver are hydrolyzed to retinol, which is bound to retinol binding protein (RBP) and transported to the target tissues (Fields, et al., 2007; Harant et al., 1993; Chao et al., 1997; Blomhoff and Blomhoff, 2006; Soprano et al., 2006). In the

circulation, RBP interacts with another protein, transthyretin (TTR), a thyroid hormone carrier (reviewed by Vieira, 1998).

Retinoids can affect the expression of many genes by modulating their transcription. Specifically, atRA is a ligand for the nuclear retinoic acid receptors (RARs) that can interact with DNA and other, co-regulatory proteins (Vieira, 1998; Fields, 2007). There is also recent interest in potential epigenetic mechanisms that are affected by retinoids and other diet-derived factors (Huang and Vieira, 2007; Love et al., 2008). Through such epigenetic mechanisms, retinoids may lead to heritable changes in DNA methylation and chromatin structure that also affect gene expression (Vieira, 2009; Hahn et al., 2008; Goo et al., 2003; Hoemme et al., 2008; Villa et al., 2007; Fazi et al., 2007; Hansen et al., 2007; Love et al., 2008; Witcher et al., 2008).

1.2.2 Retinol-binding protein (RBP)

The 21 kDa RBP is a specific circulatory vitamin A carrier protein with a binding site for all-trans retinol (Zanotti and Berni, 2004). It was first isolated from human sera (Kanai et al., 1968), and subsequently from the sera of other vertebrates—birds, amphibians, reptiles, and fish (Zanotti and Berni, 2004). In birds, for example, it is best characterized in terms of vitamin A delivery into oocytes, an important step that ensures normal development of the embryo (Vieira et al., 1995; Vieira 1998). RBP is a member of the lipocalin protein superfamily.

Lipocalins exhibit much diversity in their primary sequence and in their functions. A highly conserved tertiary structure including three conserved sequence motifs is shared by most lipocalins, (Salier et al., 2004; Flower et al., 2000; Flower, 1996). The typical structure of a lipocalin comprises 160–190 amino-acids, with 8–9 β-strands folded into a hydrogen-bonded antiparallel β-barrel enclosing an internal hydrophobic ligand-binding site (Salier et al., 2004; Flower, 1996). Most lipocalins are extracellular proteins found in vertebrates and invertebrates, plants, and bacteria (Akerstrom et al., 2000); these lipocalins possess three common functions: binding of one or more small hydrophobic molecules, binding to specific cell-surface receptors, and forming complexes with other macromolecules (Flower, 1996). These properties, for example, allow lipocalins to transfer hydrophobic ligands between cells (Flower, 1994; Zanotti and Berni, 2004). The diverse properties and functions of lipocalins also include retinol transport, invertebrate coloration, olfaction, pheromone transport, prostaglandin synthesis, bilin transport, and temperature-resistance in plants (Flower, 1996; Salier et al., 2004). Some lipocalins have implications for clinical medicine, e.g., as modulators of cell homoeostasis and of the immune response, and as carrier proteins that mobilize endogenous and exogenous compounds (Flower, 1996; Salier et al., 2004).

In the liver, the secretion of RBP from hepatocytes depends on the availability of retinol; such secretion is specifically prevented if there is a lack

of retinol, and resumes when retinol is made available (Goodman 1984; Ronne et al., 1983).

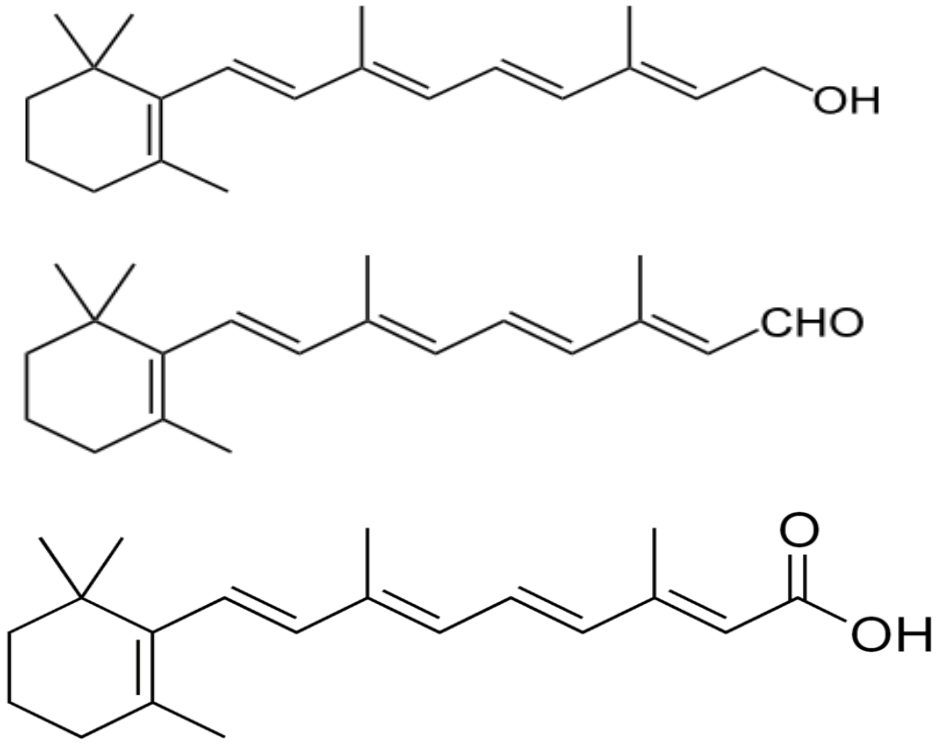

Figure 1-2 Molecular structures of some natural retinoids.

The three retinoids are shown in their all-trans forms. Retinol (vitamin A alcohol) is shown at top; retinol is the typical ligand for RBP. Retinol oxidation can produce retinal (middle), a part of the visual pigment. Retinal oxidation, in turn, can result in retinoic acid (bottom). Retinoic acid is a biologically active retinoid and can interact with retinoic acid receptor (RAR) a nuclear transcription factor.

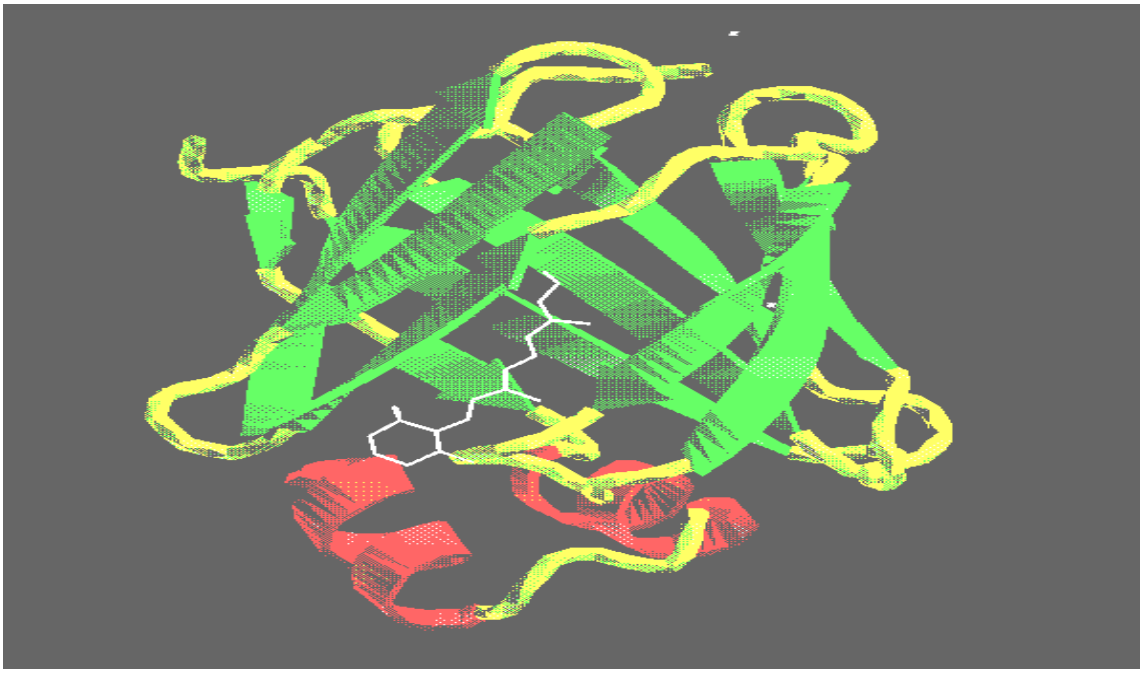

Figure 1-3 Molecular model of RBP with bound retinol.

The anti-parallel beta strands (arrows) form a cup-like structure in which the retinol (see Fig. 1-2 for retinoid structures) is bound. The protein structure also contains two alpha helices (spirals) and connecting loops or terminal strands. Other members of the lipocalin protein family also form such a cup-like structure with the potential to bind small, hydrophobic ligands. Source: University of London, ISMB, Structural Biology, Bioinformatics and Biophysics, School of Crystallography,

http://www.cryst.bbk.ac.uk/pps97/assignments/projects/rosano/vbp/rbp.gif

1.2.3 Endocytic transport – an overview of pathways

The outer animal cell membrane, the plasma membrane, controls the endocytosis (uptake/internalization) and exocytosis of many types of molecules. It is a barrier that separates the intracellular matrix or cytoplasm from the extracellular environment; and it is also a dynamic structure whose parts are continuously renewed. Some of the nutritive and regulatory small molecules, e.g., amino acids, sugars and ions, are transported across this membrane by protein pumps or channels that are integrated into the membrane. Endocytosis-mediated transport of some hormones and growth factors, of cell-surface receptors, of other particles, as well as of extracellular fluids, is known to occur in eukaryotic cells; this process typically involves invagination of the plasma membrane into the cell followed by scission and formation of membrane-bound vesicles (Conner and Schmid, 2003; Rodemer and Haucke, 2008).

Two types of endocytosis, pinocytosis and phagocytosis, comprise two categories of mechanisms that refer to the uptake of solutes and fluid and uptake of large particles, respectively (Conner and 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 and Schmid, 2003; Mukherjee et al., 1997). Pinocytosis typically refers to at least four basic

mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis (Rodemer and Haucke, 2008; Conner and Schmid, 2003). Different ligands and receptors can enter the cell through different endocytotic pathways (Conner and Schmid, 2003).

All of these endocytic events are highly controlled and influence a variety of physiological processes: e.g., hormone-mediated cellular signaling and other responses to the cellular environment, immune cell function including antigen-presentation, and homeostasis at all physiological levels (Conner and Schmid, 2003; Vieira et al., 1996).

Endocytosis may have been the first evolutionary event of vesicular trafficking (Conner and Schmid, 2003; De Duve, 1991), a way to get nutrients and to respond to the extracellular environment. Mitochondria in animal cells and chloroplasts in plant cells were likely obtained by phagocytosis of their respective prokaryotic precursors (Conner and Schmid, 2003).

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 (PIP2)-enriched sites at the plasma membrane and deliver their cargo to the endosomal compartments (Galli and

Haucke, 2004; Schweizer and Ryan, 2006; Krauss and Haucke, 2007; Di Paolo and De Camilli, 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 and 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 (Rodemer and Haucke, 2008; Kirchhausen, 2000). Additionally, 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 et al., 1995; Yarar et al., 2005). The GTPase dynamin participates in the fission of the nascent clathrin-coated vesicle from the plasma membrane (Conner and Schmid, 2003). Adaptor and accessory proteins can bind the phospholipid PtdIns(4,5)P2 that accumulates at sites of CME (Ford et al., 2002, 2001; Beck and Keen, 1991; Zoncu et al., 2007).

Among the adaptor proteins, the AP-2 complex participates in cargo selection and recruitment of accessory proteins (Robinson and Bonifacino, 2001); there is genetic (Mitsunari et al., 2005; Gonzalez-Gaitan and Jackle, 1997), biochemical (Praefcke et al., 2004; Edeling et al., 2006), and cytological (Motley et al., 2003; Hinrichsen et al., 2003; Huang et al., 2004) 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 (Lewin and Mellman, 1998; Traub, 2005; Lakadamyali et al., 2006; Haucke, 2006).

1.2.3.2 RME: uptake of nutrients and their carrier proteins

Receptor-mediated endocytosis (RME) allows extracellular ligands to be captured and concentrated on clathrin-coated pits and in clathrin-coated 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 and Schmid, 2003; see also discussion of HRP in Section 3). In RME, the extracellular ligand binds to its complementary membrane receptor(s), and then the plasma membrane region containing the receptor-ligand complex enters the cell, typically by CME (Lodish, et al., 2000; Alberts et al., 2002). 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).

Twenty five or more different receptors participate in RME through the clathrin-pathway; and some of these receptors, e.g. for Tf (TfR) and LDL (LDLR), enter the CME pathway regardless of whether ligand is present or not (Alberts et al., 2002). Other receptors, e.g. for growth factors such as EGF, are recruited to this pathway only when bound to their specific ligand(s) (Alberts et

al., 2002). RME can serve various purposes such as generation of cellular signalling, e.g., EGF-EGFR, and transport of nutrients, e.g. iron-Tf-TfR (Shankaran et al., 2007). In the context of evolution, the *co-evolution* of ligands and their specific receptors presents an interesting model (Moyle et al., 1994); for example, a receptor mutation may result in affinity for a new ligand or increased affinity for one of its existing ligands, or a mutation in either the receptor or ligand can result in loss of transport or signalling activity.

Some of the studies reported in this thesis involve the nutrient carrier Tf, a circulatory iron transport protein. Tf is often used as a standard in endocytic transport studies because its receptor-mediated endocytosis is well characterized (see also discussion in Chapter 5; Abe et al., 2008; Johnson et al., 2007; Oshiro et al., 1993; Ponka and Lok, 1999). Serum Tf is part of an iron-transport protein family of proteins that includes ovotransferrin in oocytes, and lactoferrin in milk (Daniels et al., 2006). The transferrin receptor (TfR, CD71) is an essential protein required for iron uptake, regulation of cell growth and cell survival (Aisen, 2004; Morgan et al., 1986; Young et al., 1985; Kang et al., 2005; Carosio et al., 2007). Diferric Tf binds to TfR and both are internalized through clathrin-coated pits.

1.2.3.3 RME of retinol and RBP

The existence of cell surface RBP receptors has been suggested by many studies (e.g., Redondo et al., 2008; Blaner, 2007; Kawaguchi et al.,

2007; Huang and Vieira, 2006; Sundaram et al., 1998; Heller 1975; McGuire et al., 1981; Bok and Heller, 1976; Smeland 1995; Rask and Peterson, 1976; Senoo et al., 1990; MacDonald et al., 1990; Vahlquist et al., 1992; Creek et al., 1989; Hodam et al., 1991; Fortuna et al., 2003; Mansouri et al., 1998; Sivaprasadarao and Findlay, 1988; Senoo et al., 1993; Hagen et al., 1999); in some, but not all of these studies, there is evidence for RBP endocytosis. Possible recycling of endocytosed RBP has, to date, not been reported. Cells may use different mechanisms for the uptake of RBP and vitamin A, as may be the case with some other vitamins and other regulatory factors (cf., Vieira et al. 1996; Prasad and Ganpathy, 2000; Ritter et al., 1995).

Most recently, STRA6 (stimulated by retinoic acid gene 6), an integral membrane protein with several transmembrane domains has been identified as a mediator of retinol uptake from RBP (White et al., 2008; Kawaguchi et al., 2008a; Kawaguchi et al., 2008b; Wolf, 2007; Blaner, 2007; Kawaguchi et al., 2007). STRA6 from retinal pigment epithelial (RPE) cells can bind to RBP with a high affinity (K_d = 59 nM) and leads to uptake of vitamin A from the vitamin A-RBP complex (Blaner, 2007). Expression of STRA6 can dramatically increase retinol uptake from RBP by COS-1 (CV-1 simian cells transformed by origin-defective mutant of SV40 which codes for wild-type T antigen) cells. RNA interference (RNAi) knockdown of STRA6 in WiDr cells (a human colon carcinoma line) has an opposite effect of decreasing retinol uptake (Blaner, 2007). Other recent studies, however, suggest that STRA6 may not only mediate the uptake of retinol into cells, but also the efflux of retinol from cells;

and, thus, protect cells from excessive retinoid accumulation (Isken et al., 2008). In addition to expression on the basolateral membrane of RPE cells, STRA6 has been found to have strong expression in the blood vessels of the retina, the hippocampus and spleen (Blaner, 2007). Mutations in human STRA6 result in various severe pathological phenotypes such as anophthalmia/microphthalmia (White et al., 2008), mental retardation, congenital heart defects, lung hyperplasia, diaphragmatic hernia, alveolar capillary dysplasia, and others (Pasutto et al., 2007; Golzio et al., 2007; Kawaguchi et al., 2008b); and these diseases are thought to be due to abnormal cellular levels of retinoids. Further research should help clarify the relation between such diseases and the transport and metabolism of retinol.

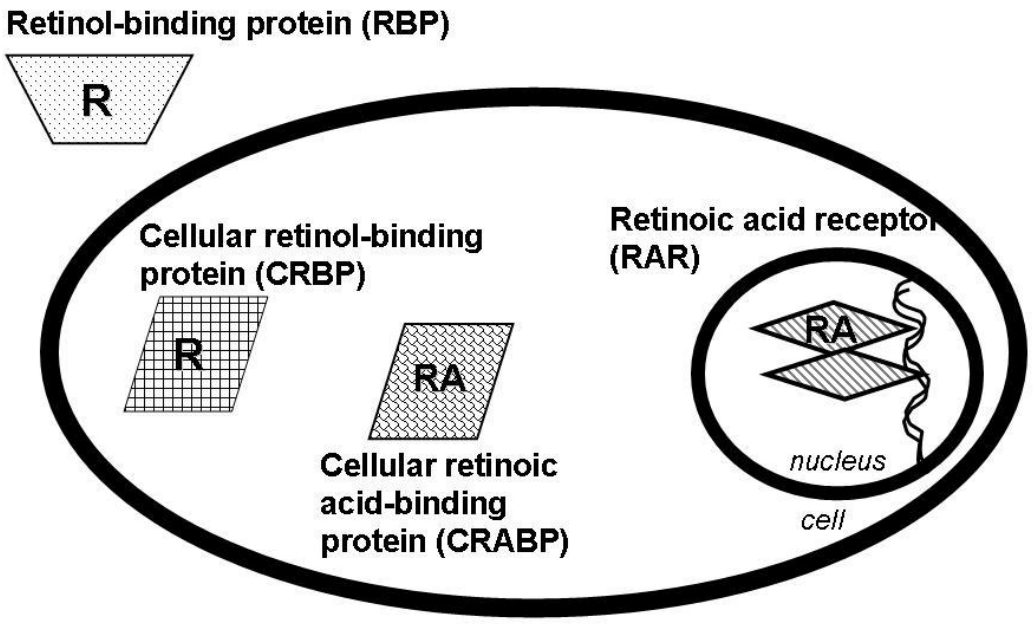

Figure 1-4 Schematic of different types of retinoid-binding proteins and their locations.

Each protein type can have different isoforms (not shown).

1.3 Main hypotheses tested

The following are the main hypotheses being tested in relation to the above objectives:

(i) It is hypothesized that an RBP receptor exists on the surface of human epithelial A431 cells and mediates RBP endocytosis;

(ii) It is hypothesized that, as with other nutrient carrier proteins such as transferrin, RBP recycles out of the cell after endocytosis;

(iii) It is hypothesized that, as suggested by studies of age-related changes in endocytic transport efficiency, tissues from old mice are less efficient in terms of accumulating exogenous RBP relative to the same tissues of young mice.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and biochemical reagents

Human holo-transferrin (Tf), biotinylated Tf (bTf), streptavidin-peroxidase (streptavidin-HRP or ESa), 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. Protease inhibitors were purchased from Roche. Dulbecco's modified Eagles medium (DMEM) and fetal bovine serum (FBS) were obtained from different sources: Invitrogen, Gibco, and Sigma-Aldrich. Phosphate-buffered saline (PBS) was obtained from Gibco.

The ligand-enzyme conjugates (ESa-bTf) were prepared by incubating ESa with bTf at a ~ 1:1 molar ratio in PBS for 1 h at room temperature, typical incubation conditions previously reported, e.g., (Shimada et al., 2005; Kamemura and Kato, 1998; Weitz-Schmidt et al., 1996; Vieira 1998; Graf and

Friedl, 1999), for the formation of stable ESa-bX complexes (X = protein or other biomolecule/material). Preparation of b-RBP as well as other methods, chemical reagents, and equipment are detailed below in the relevant sections.

Phytochemical plant extracts (used to illustrate the use of the endocytosis screening assay, Section 5) were prepared by re-suspending 2 mg of dry plant material per ml of water. The extracts were vigorously vortexed and insoluble material was removed by centrifugation. A project is underway in the laboratory to standardize a large number of the extracts in terms of total polyphenol content. At this point, different (i.e., not total polyphenol content) standardizations are available for two of the extracts analyzed in Section 5; these are as follows: the *Ginkgo biloba* material was standardized for content of total flavonoid glycosides, 24% by weight; the *Vaccinium myrtillus* extract was standardized for total anthocyanin content,12.5 μg/ml.

2.2 Cells, cell preparations and animals

2.2.1 Cells

The human epithelial (keratinocyte, human epidermoid squamous carcinoma) cell line, A431, 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 2.3.3 below), cells were cultured in 96-well plates (Sarstedt).

2.2.2 Cell preparations

For the preparation of semi-intact cell membranes (SICM), A431 cell lines 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 half an hour in serum-free medium. They were then gently washed with 1 ml ice-cold PBS and 1 ml ice-cold KSHM (100 mM potassium acetate, 85 mM sucrose, 20 mM HEPES and 1mM magnesium acetate). Cells were then scraped with a rubber tool and collected into 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 14,000 rpm, 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 1mM magnesium acetate) and stored frozen at -80°C.

For binding assays, equal volumes of SICM were aliquoted into tubes, each aliquot performed immediately after resuspension to ensure equal membrane amounts.

2.2.3 Animals

Work with animals was approved by the Simon Fraser University Animal Care Committee. For the animal injection and tissue preparation experiments, C57BL/6 male mice were used. Young animals were 1-2 months of age, purchased from Charles River Laboratories (USA); old animals were approximately 28 months of age, purchased from NIH's National Institute of Aging (USA). The animals were injected with biotin-labelled RBP into the tail vein. Maximum injected volume was 100 μl and contained 2 μg of b-RBP. Animals were euthanized by carbon dioxide asphyxiation, and then perfused with physiological saline; these procedures were performed by the animal care facility personnel of the University.

2.3 Biochemical and cellular methods

2.3.1 Ligand biotinylation

Biotinylated RBP (b-RBP) was prepared by incubating biotinylation reagent, BNHS (Biotin N-hydroxysuccinimide ester; Sigma), with RBP at a molar ratio of 5:1. 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. The b-Tf used was purchase from Sigma-

Aldrich. b-RBP and b-Tf were detected by blotting (e.g., Section 2.3.2) or ELISA (e.g., Section 2.3.3) procedures using streptavidin-HRP.

2.3.2 Streptavidin-peroxidase blotting

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was placed in a blocking solution, 5% non-fat milk powder in TBST (TBST, tris-buffered saline with 0.1% Tween 20) for half an hour at room temperature, with gently shaking. The blocking solution was removed and the membrane was probed with streptavidin-HRP (1:2000 dilution) in fresh blocking solution, with gently shaking overnight at 4°C, or 5 hours at room temperature. The membrane was rinsed once with TBST, and then washed twice in TBST with gentle shaking, for 5-10 min each time. HRP activity was then detected with enhanced chemiluminescence (ECL) reagents (Amersham Biosciences/GE Healthcare).

2.3.3 Endocytosis assays

Cells were cultured on 96-well plates to the same confluency in all wells, and then incubated in serum-free medium, rinsed two times with PBS, and incubated with (i) ESa-bTf (ligand-enzyme conjugate), (ii) ESa alone (control), (iii) ESa-bTf + 50 molar excess of Tf (control), (iv) ESa + 50 molar excess of Tf (control).

Each incubation occurred either in 100 μl PBS-SFM (Method A; a 1:1 volume mixture of PBS, and serum-free DMEM) or 100 μl PBS-SFM-BSA (Method B; a 1:1 volume mixture of PBS containing 0.2% BSA, and serum-free DMEM) for 10 min at 37°C. Cells were washed two 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.

Internalization of conjugates was measured spectrophotometrically after a peroxidase reaction involving 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 color reactions were terminated with 4 N sulfuric acid. Absorbance at 492 nm was measured (Statfax-2100 ELISA plate reader).

For testing potential inhibitory or stimulatory activities of phytochemicals (or purified compounds/drugs), the substances were added immediately before the ligand at the concentrations indicated in the figures (Section 5). Effects of more long-term exposure were also tested; cells were pre-incubated with these compounds for a period of 4 hours before the addition of biotin-ligand.

2.3.4 Recycling assays

Cells were cultured on 96-well plates to the same confluency in all wells (~80%), and then incubated in serum-free medium, rinsed two times with PBS, and incubated with ligand-enzyme conjugate, either ESa-bTf or ESa-bRBP, in 100 μl PBS-SFM-BSA (a 1**:**1 volume mixture of PBS containing 0.2% BSA, and serum-free DMEM) for 10 min at 37°C. Cells were washed two times with ice-cold acidic buffer (25 mM acetic acid and 100 mM NaCl, pH 3), followed by neutralization with PBS. After removal of PBS from the last rinse, 20 μl 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) buffer was added to control wells in order to quantify total internalized ligand. Then 0.1 ml pre-warmed 37°C PBS-SFM-BSA (PBS:SFM at a ration of 1:1, with 0.02% BSA) was add to the remaining (non-control) wells and the cells were incubated for an additional period of 10-30 min.

The plate was subsequently placed on ice and the medium was collected from each well. The cells were then gently washed twice with the low pH, neutralized with PBS, and lysed with detergent solution as above. The OPD-based colorimetric reaction was performed as in Section 2.3.3 above. Loss of internalized ligand (potential recycling) was expressed as percent of total internalized (control above) remaining after the second 37°C incubation.

2.3.5 Chemical cross-linking of b-RBP and cell membranes (SICM)

One tube of frozen A431 SICM (preparation method in Section 2) was thawed on ice and centrifuged for 1 min at 14,000 rpm (Spectrafuge 16M). The supernatant was removed, and pellet rinsed with 1 ml PBS by mixing, followed by re-centrifugation as above. The rinsed pellet was re-suspended by pipetting (cut-tip pipette) in an equal volume (typically ~100 μl) of PBS and divided equally into two Eppendorf tubes. RBP at 2.5 μg/μl was added to one of the tubes (50-fold competition control), followed by the addition of b-RBP, 0.05 μg/μl, to both tubes. The tubes were vortexed for 5 s and incubated overnight at 4°C with gentle mixing (rotating platform). The membranes were then centrifuged, 30 s at 14000 rpm, and the pellet was rinsed with PBS, and resuspended in PBS as above. The tubes were vortexed 5 s twice again and 3 μl of 5% glutaraldehyde was then added to each tube. Both tubes were immediately vortexed six times and placed on ice. To each tube 4× Laemmli Buffer (LB; 1× Laemmli buffer composition: 60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% 2-mercaptoethanol and 0.002% bromophenol blue) was added to make 1× final concentration. The contents were heated at 95°C for 5 min to denature the proteins, and cooled down at room temperature before loading onto gels for SDS-PAGE.

2.3.6 Mouse tissue analysis for b-RBP

Kidney, lung and liver tissues from young (1.5 months of age) and old (28 months of age) C57BL/6 mice injected with b-RBP 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 15,000 rpm for 15 min at 4°C. The resulting supernatant was removed and loaded onto a 96-well plate (Nunc) pre-coated with 4.8 µg/ml anti-RBP IgG in 50 mM sodium bicarbonate buffer (pH 9.6), and then incubated overnight at 4°C. The wells were then rinsed with PBS, ST (blocking) buffer, and three times again with PBS, followed by addition of 1 µg/ml streptavidin-HRP in 200 µl blocking buffer and an incubation overnight at 4°C or 3 hours at room temperature. After a rinse with PBS ×1, a wash with ST (Blocking) buffer ×1 and rinse again with PBS ×3, a color reaction was performed using o-phenylenediamine (OPD, Sigma), chromogenic solution (50 mM Na_2HPO_4, 30 mM sodium citrate, pH 5) and 30% H_2O_2. The color reaction was stopped by adding 2N H_2SO_4. The resulting color intensity of the solution in the wells was quantified on a StatFax 2100 absorbance plate reader (Awareness Technologies) at 490 nm (with 650 differential filter) to assess biotin-ligand levels.

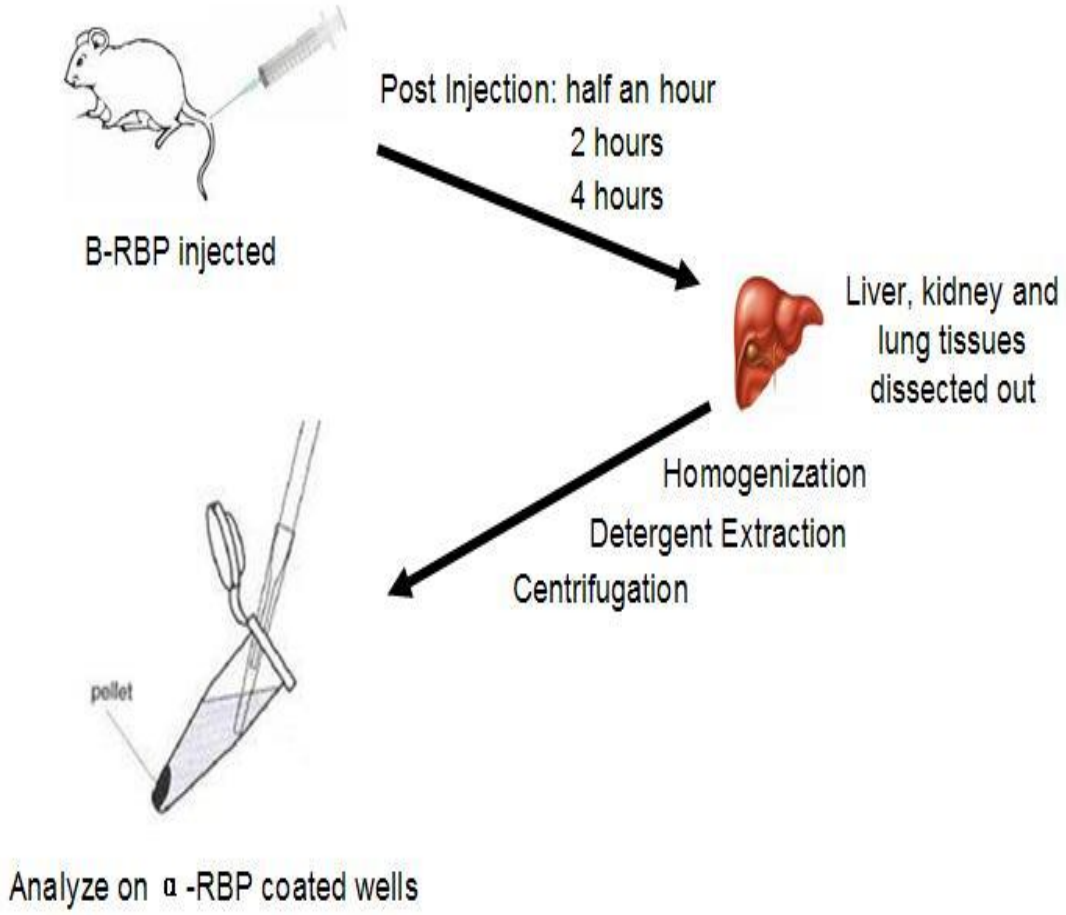

Figure 2-1 Flowchart of tissue accumulation analysis procedure.

Biotinylated RBP (b-RBP) was injected into the tail vein of the mouse. At the designated time points post-injection, the liver, kidney, and lung tissues were dissected out, extracted with detergent and analyzed for b-RBP content on wells coated with anti-RBP (α-RBP) IgG. For this thesis project, the half-hour time point was most relevant to endocytic transport.

2.3.7 Analysis of b-RBP binding to crude membranes prepared from tissues of young and old mice

Tissue samples from young (1.5 months) and old age (28 months) mice (non-injected animals) were homogenized as described above for the injected tissues. After homogenate centrifugation the supernatant was discarded and the pellet was re-suspended in two volumes of PBS (a small aliquot was removed for protein determination). Bovine serum albumin (BSA) was added to give a final BSA concentration of 0.02%. The resulting PBS-BSA extract was divided into three 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 RBP (b-RBP) was added to each sample and samples were incubated overnight at 4°C with gentle mixing. The extracts were centrifuged at 15,000 rpm for 30 s at 4°C, followed by a pellet re-suspension with a PBS-BSA solution containing 0.02% BSA. The extract was re-centrifuged, and the resulting pellet was rinsed with PBS and then re-suspended with blocking buffer (pH 7.4). This extract was plated onto a 96-well plate (Nunc) pre-coated with 4.8 μg/ml anti-RBP IgG in 50 mM sodium bicarbonate buffer (pH 9.6), and then incubated overnight at 4°C. After rinses and streptavidin-HRP probing as above, an OPD color reaction (see above for details) was performed to assess the captured b-RBP.

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, paired (unless noted otherwise) Student's t-tests were performed on the data to compare a given experimental treatment with a reference or 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 * or **, respectively, for statistical significance (alpha level, 0.05).

CHAPTER 3: RECEPTOR-MEDIATED ENDOCYTIC TRANSPORT OF NUTRIENT CARRIER PROTEINS: RBP

3.1 Introduction

The potential mechanisms by which RBP mediates cellular retinol uptake are controversial and, in many cases, not well defined. Characterization of RBP receptors is of fundamental importance in terms of studying these mechanisms. Experiments were carried out in an attempt to further characterize RBP receptors and the post-endocytic fate of RBP in a human epithelial cell line (A431 keratinocytes). Keratinocyte-type cells are known to have a high RBP binding capacity relative to other cell types (Smeland et al., 1995); they represent a good experimental model for such receptor and transport studies. Previous data from Scatchard analysis of bound RBP have indicated the presence of two high-affinity RBP binding sites on the A431 epidermal cells (Huang and Vieira, 2006). Table 1 provides a

comparison of RBP receptor studies in these and other cells. This study was carried out in order to obtain information regarding the possible size (molecular weight) of the RBP receptor in these A431 keratinocytes, and to provide the first indication (in any cell type) of the fate of intracellular RBP after endocytosis. In relation to the latter experiments, it was hypothesized that, as with other nutrient carrier proteins such as transferrin, RBP recycles out of the cell after endocytosis.

Both the receptor and recycling studies have the potential to provide novel and important basic information about the physiological transport of RBP. Knowledge about the receptor, for example, is necessary if, ultimately, one is interested in modulating RBP or retinol delivery to cells. Based on current knowledge regarding (a) RBP levels and diseases related to obesity and insulin resistance (see discussion in Section 3.3), and (b) the effects of retinol metabolites on cell proliferation and differentiation, such modulation of transport may represent an important therapeutic approach, e.g., for some metabolic diseases and cancers. Moreover, a novel strategies based on delivery of therapeutics through receptor-mediated endocytosis of RBP may be applied to the treatment of some liver and other diseases (e.g., liver cirrhosis, Sato et al., 2008; see also Section 5.1).

Table 3-1 Comparison of some biochemical parameters, Kd (receptor-ligand affinities) and Bmax (total number of ligand binding sites), for RBP receptor activities in different types of cells.

Cell type	**K_d**	**B_{max}**
Keratinocytes (A431)[1]	0.7 nM 30 nM	12000 sites/cell 82000 sites/cell
Bovine pigment epithelium[2]	5 nM	52000 sites/cell
Placental cells[3]	37.4 ±12.9 nM	1122 ±138 fmol RBP bound/mg of protein
Bone-marrow cells[3]	46.1 ± 5.3 nM	930 ± 18 fmol RBP bound/mg of protein

Kidney cells[3]	47.6 ± 19.2 nM	619 ± 80 fmol RBP bound/mg of protein
Liver cells[3]	32.5 ± 15.3 nM	295 ± 66 fmol RBP bound/mg of protein
Lipocytes[4]	4.93 nM 234 nM	0.134×10^6 sites/cell 3×10^6 sites/cell
Myofibroblasts[4]	246 nM	0.33×10^6 sites/cell
Placental membrane cells[5]	3.0 ± 2.7 nM 95 ± 35 nM	83 ± 8.6 fmol of RBP bound/50 µg of membrane protein 8.0 ± 4.8 fmol of RBP bound/50 µg of membrane protein

Parenchymal liver cells[6]	12.7 ± 3.2 nM	138 ± 19 fmol/10^6 parenchymal cells
J774 macrophages[7]	1.9 nM	480 fmol/mg protein

[1]Huang and Vieira, 2006; [2]Heller, 1975; McGuire et al., 1981; [3]Smeland 1995; Rask and Peterson, 1976; [4]Fortuna et al., 2003; [5]Sivaprasadarao and Findlay, 1988; [6]Senoo et al., 1993; [7]Hagen et al., 1999.

3.2 Results

3.2.1 Characterization of RBP receptors

To identify and characterize (e.g., determine molecular weight) of potential cell membrane RBPRs (RBP receptors), chemical cross-linking of biotin-labelled RBP (b-RBP) with the membranes was performed (Methods in Section 2.3). Streptavidin-HRP blotting was used to detect the covalent bRBP-RBPR complex. (The streptavidin component binds biotin with very high affinity, approximately 10^{-15} M (Singh et al., 2005)). Molecular weight(s) of RBPR were calculated by subtracting the known molecular weight of RBP (21 kDa) from that of the whole complex. Moreover, if the receptor is specific for RBP, it should be competed by an excess of unlabelled RBP.

The results of ligand-receptor chemical crosslinking are shown in Figure 3-1, a molecular complex of about 178 kDa that includes the 21 kDa b-RBP. Assuming a 1:1 ratio of b-RBP to its putative receptor, the calculated molecular weight of this membrane receptor would be about 157 kDa. Competition with excess non-biotin-labelled RBP (Figure 3-1, lane 2) indicates that the receptor is specific for RBP. The biotin-labelled RBP (b-RBP) itself, without membranes, does not yield molecular complexes in the 170-180 kDa region (without membranes).

Figure 3-1 Cross-linking of A431 cell membranes with b-RBP.

A b-RBP-containing molecular complex of about 178 kDa is revealed (lane 1) and is competed by a 50-fold molar excess of unlabelled RBP (lane 2). Positions of molecular weight markers are shown, from top to bottom: 200.0 kDA, 116.3 kDa, 97.4 kDa, 66.2 kDa.

3.2.2 Endocytic trafficking of RBP

In addition to RBP receptors, the human keratinocyte line A431 also expresses abundant levels of Tf receptors at the cell membrane (Vieira 1998; Castagnola et al., 1997; Hopkins and Trowbridge, 1983; Wiley, 1988). Both the RBP and Tf receptors lead to internalization of their respective ligands in these cells (Huang and Vieira, 2006 and references therein; Azizi and Wahl, 1997; Hopkins and Trowbridge, 1983; Wiley, 1988). It is well known that internalized Tf delivers its iron to the cell and then recycles back to the cell surface along with its receptor. The fate of the internalized RBP, however, has not previously been examined. To study this aspect of RBP endocytic trafficking, cells were pre-loaded with b-RBP and then its loss from intracellular compartments was quantified. If there is a loss, it may be due to b-RBP degradation in the cell or to recycling of the internal b-RBP back out of the cell (or a combination of both). To obtain evidence for possible RBP recycling back out of the cell, after the removal of any remaining exogenous b-RBP, the cell medium was analyzed for re-appearance of the labelled intracellular RBP (b-RBP).

Figure 3-2 shows that about half of the total amount of b-RBP that accumulated inside the cell was lost after a 10 min period at 37°C. In comparison, about 40% of the b-Tf load was lost in this time period, a process know to be the result of b-Tf recycling back out of the cell (see above). Free

HRP, a fluid phase marker (non-receptor-mediated endocytosis), was eliminated more quickly, about 70% of its accumulated load was lost in the 10 min period.

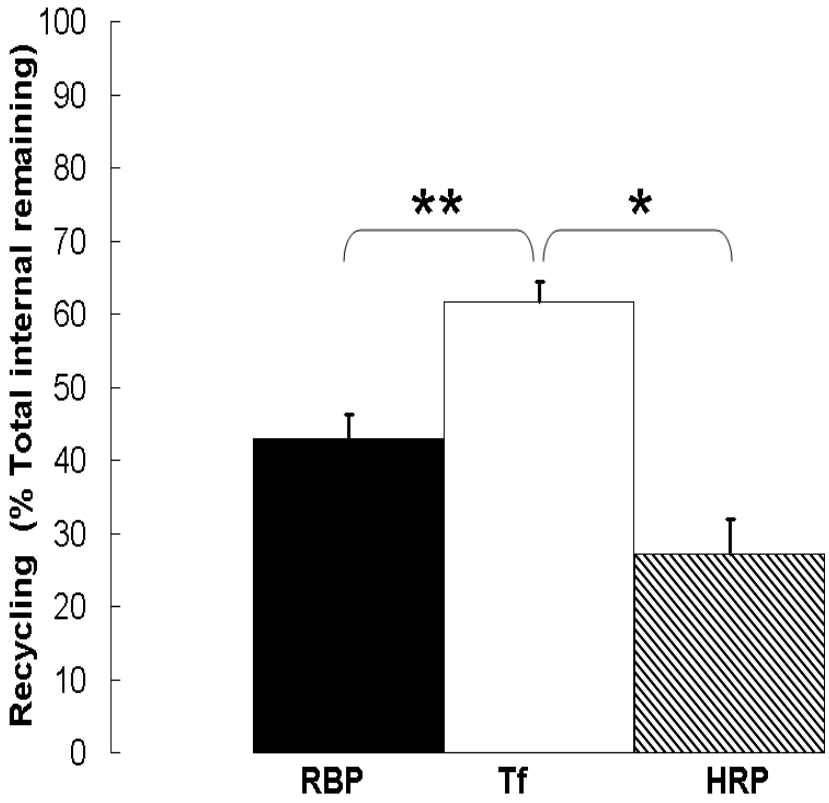

Figure 3-2 Loss of the intracellular pool of endocytosed, exogenously added b-RBP, b-Tf, or HRP.

The post-loading incubation time was 10 min., 37°C. Asterisks indicate statistically significant differences relative to the Tf standard, $p < 0.05$ (defined in Section 2.4). HRP, horseradish peroxidase, is a plant enzyme and fluid phase marker.

At least some of the b-RBP loss shown in Figure 3-2 can be accounted for by recycling of b-RBP out of the cell and into the medium. Figure 3-3 shows that such recycled b-RBP could be detected in the medium. At this point it is not possible to quantify the percentage of total internal b-RBP that is recycled and the percentage, if any, that is degraded.

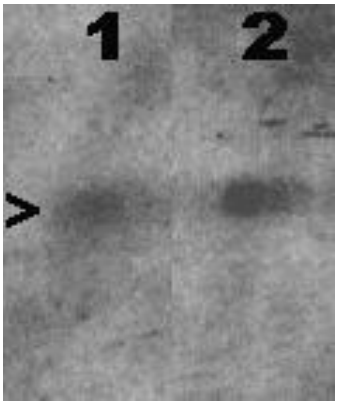

Figure 3-3 Streptavidin-HRP blot of recycled b-RBP in medium.

The recycling time was 10 min, 37^{o}C. Lane 1 represents the signal in the medium, and lane 2 is the signal from a b-RBP positive control.

3.3 Discussion

The results above, together with those previously published on the A431 keratinocyte cell line (Huang and Vieira, 2006), provide evidence for receptor-mediated transport of RBP that could effect retinol delivery into cells. As discussed further below, this transport shares properties with some other nutrient delivery mechanisms, e.g., Tf.

Biochemical evidence for cell surface RBP receptors has been obtained using a variety of cell lines, e.g., Table 3-1. The current study provides the first size characterization of an RBP receptor, a molecular species of about 157 kDA. STRA6, a novel protein with several transmembrane domains has been recently identified and reported as a retinol transporter that can interact with RBP (see Introduction for more information on STRA6 and related references). Interestingly, in the original RBP-membrane crosslinking experiments performed by Kawaguchi et al. (2007), a specific, minor RBP-containing species of about 180 kDa was observed along with the major ~80 kDa STRA6-RBP complex. The RBP complex that I observed was also about 180 kDa. Whether these 180 kDa species represent RBP-STRA6 multimers, e.g. dimers, or a different RBP receptor is not known at present.

After receptor-mediated endocytosis, transferrin releases its ligand, iron, into the acidic endosome; the empty Tf then recycles back out of the cell

bound to its receptor (Hopkins and Trowbridge, 1983). Other ligands such as LDL release from their receptor, and the receptor recycles back to the cell membrane; but most of the ligand is eventually degraded in lysosomes (Goldstein et al., 1981). In the case of internalized RBP, nothing was known about its fate prior to the current study. This study provides the first evidence, in any cell type, that the internalized RBP signal is rapidly lost from the cell (about half is lost in 10 min, Fig. 3-2, in this epithelial line); and at least some of this loss can be accounted for by recycling of the internal RBP back out of the cell (Fig. 3-3) . The loss of RBP signal in the 10 min time period was significantly greater than the loss of Tf (Fig. 3-2). This result suggests that the RBP is recycled more quickly than Tf out of the cell, or that some of the internalized RBP (unlike the case with Tf) is degraded. It is also possible that the recycled Tf is re-internalized from the medium more efficiently than the recycled RBP; this possibility may be less likely because of the dilution of recycled Tf in the medium and the short recycling time of 10 min. Interestingly, of the three markers that were examined, RBP, Tf, and HRP, the latter exhibited the greatest loss in the 10 min period. A literature review didn't provide any evidence for receptor-mediated endocytosis of HRP; rather, it is likely internalized non-specifically along with the fluid phase. The result that most of the HRP is lost from the cell in that short time period may, in turn, suggest that the presence of a specific receptor (i.e., for RBP or Tf) prevents the rapid degradation or recycling (or both) of its ligand. This result may also suggest that non-specifically internalized extracellular fluid components that

are not needed by the cell are rapidly returned to the extracellular medium, or rapidly degraded.

If one assumes that the well-established Tf recycling kinetics are applicable to RBP (at present there is no evidence for or against this), one could estimate based on Figure 3-3 that, of the approximately 55% internal b-RBP lost in the 10 min period, about 15% is degraded and 40% (similar to Tf) is recycled back out of the cell. Future work should help to further establish RBP recycling and, for example, to determine if internalized holo-RBP delivers its retinol cargo to the cell and recycles back out of the cell empty, in the apo-RBP form. If so, this would be similar to the transferrin paradigm.

Recent evidence suggests that RBP can function not only in vitamin A delivery but also as a signalling molecule with more general effects on metabolism (references below). RBP (referred to as RBP4 in these studies) is produced by adipose tissue (as well as liver and others) and may function as an adipokine—signalling between adipocytes and other cell types; it may influence inflammation, insulin resistance and the risks of obesity and cardiovascular diseases (Chavez et al., 2009; Erikstrup et al., 2009; Motani et al., 2009; Matsuzawa, 2006; Broch et al., 2009; Ingelsson et al., 2009; Wu et al., 2009). At present there is much research, and controversy, in terms of these other physiological and pathological functions of RBP. The current RBP transport results provide a base for future studies on the possible contribution that RBP recycling may have to such RBP-dependent signalling and metabolic regulation. Modulation of the receptor-mediated uptake of RBP, e.g. through

the use of factors that influence endocytic trafficking (cf. Section 5) or RBP-receptor interactions, may also have an influence on such physio-pathological regulation.

CHAPTER 4: EFFECTS OF AGING ON RBP TISSUE ACCUMULATION LEVELS AND RBP BINDING TO MEMBRANE PREPARATIONS

4.1 Introduction

Aging is know to affect the efficiency of various physiological transport processes including endocytic trafficking and receptor-mediated endocytosis (RME) (Park, 2002; Park et al., 2002; Cnop et al., 2000; Dini et al., 1996; Verbeke et al., 1996; Blanpied et al., 2004; Cessac et al., 1993; Haq and Szewczuk 1992; Ito et al., 2007; Le Couteur et al., 2008 and references therein). Blanpied et al. (2004), for example, have provided evidence that aging decreases endocytic efficiency of Tf (clathrin-mediated) in the dendrites of hippocampal neurons. There is also evidence for decreased rates of Tf RME in aged animals (Chen et al., 2009; Morgan and Moos, 2002). Possible effects of aging on RME of RBP and vitamin A uptake are not well understood.

In the experiments detailed below, accumulation of injected b-RBP was analyzed in various mouse tissues from young and old animals. Such tissue accumulation does not provide direct indications of RME and endocytic efficiency in the cells, but may provide a reliable estimate (see below and Sections 4.2.3 and 4.3) of such parameters. Experiments directed at analyzing possible changes in RBP-binding efficiencies with age were also performed. The combination of the binding and accumulation data was used for a comparative estimation of possible endocytic efficiencies in the A431 keratinocytes and other cells. Overall, based on previous studies of age-related changes in endocytic transport efficiency, it is hypothesized that tissues from old mice are less efficient in terms of accumulating exogenous RBP relative to the same tissues of young mice.

4.2 Results

4.2.1 Tissue accumulation levels of b-RBP

Accumulation of injected, b-RBP in some tissues of young and old mice was tested at ½ h P.I. (post-injection) using Enzyme-Linked Immunosorbent Assay (ELISA) and colorimetric analysis. Each tissue extract was prepared at a concentration of 0.5 g/ml (see Materials and Methods). Figure 4-1 shows the averages of all young (Y) and old (O) tissue data points and indicates lower

accumulation in the older liver and kidney tissues. Lung tissues displayed much greater variation and a minor difference between Y and O when all data was pooled; much less variation was observed with the *paired* ratios (see below, Figure 4-2) especially for lung tissues. When the results from each experiment were paired and O/Y ratios were calculated, Figure 4-2, there was evidence for lower accumulation in all examined tissues from old mice relative to those from young animals. In comparing the data sets for a given young and old tissue, the only difference between the two ages that attained statistical significance was that for kidney. Accumulation in lung was least influenced by aging, and displayed other unique features (discussed further in relation to Figure 4-3).

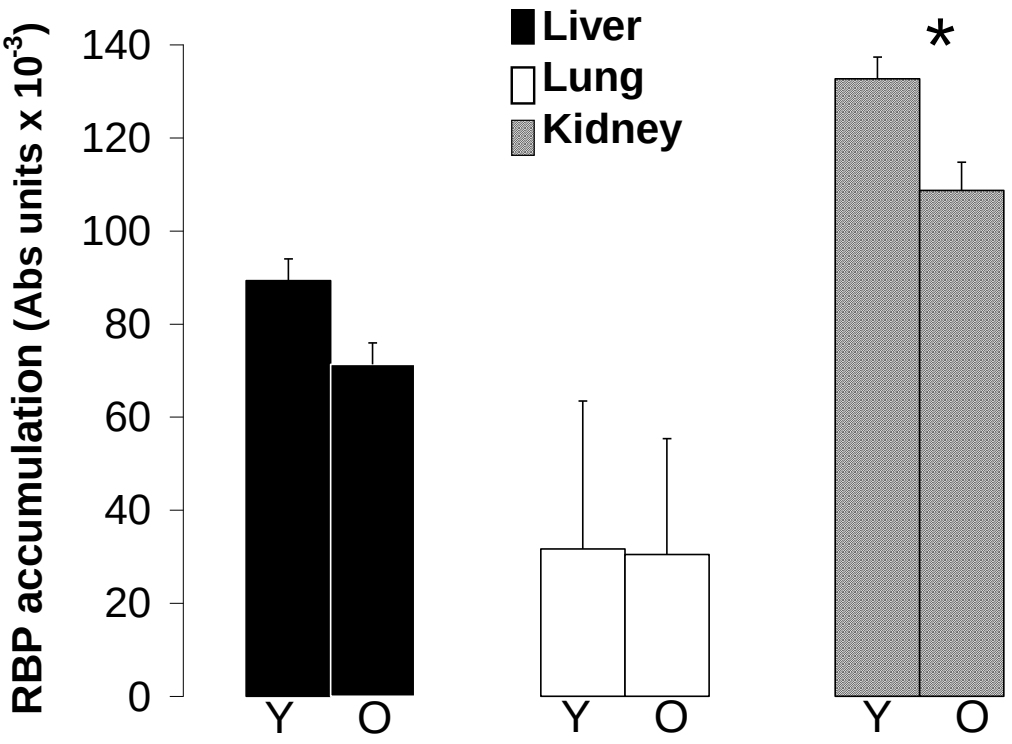

Figure 4-1 In vivo accumulation of b-RBP 30 minutes after intravenous injection into young and old mice.

Biotinylated RBP (b-RBP) was injected into young and old mice. After 30 min post-injection, tissues were removed and extracts were prepared (0.5 g/ml). These extracts were analyzed for b-RBP levels via colorimetric measurements after binding of streptavidin-peroxidase. Results for each young and old tissue were pooled and the averages ± SEM are shown (n = 6). A statistically significant difference was observed for kidney tissue (* $p < 0.05$).

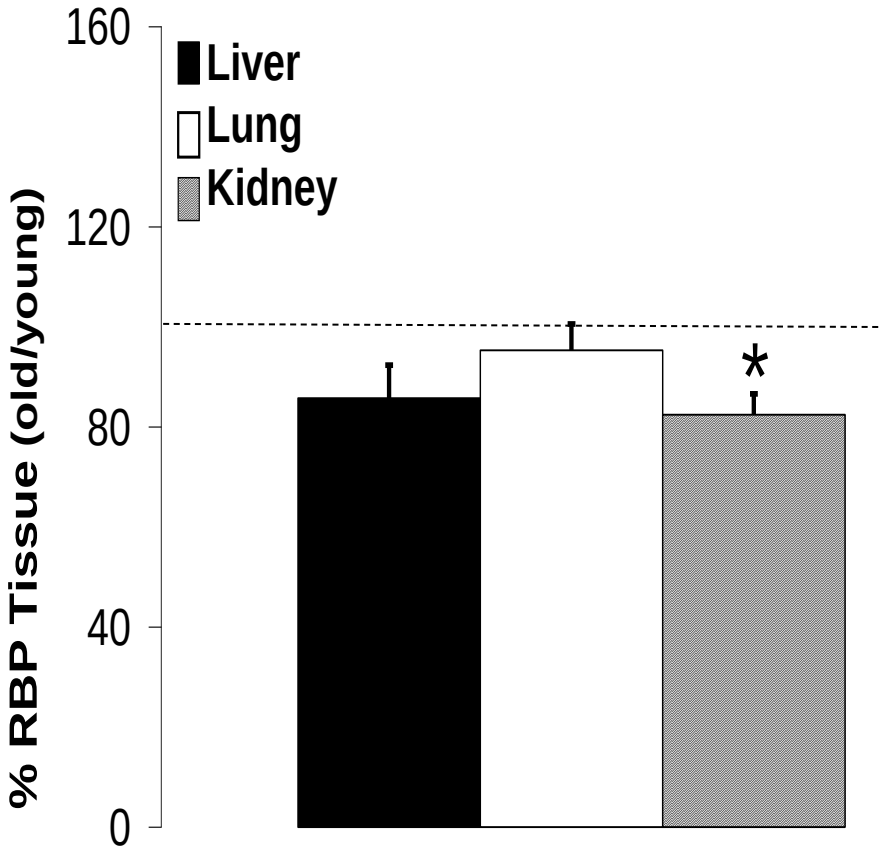

Figure 4-2 Relative in vivo accumulation of b-RBP 30 minutes after intravenous injection into young and old mice.

Biotinylated RBP (b-RBP) was injected into young and old mice. After 30 min post-injection, tissues were removed and extracts were prepared (see Materials and Methods). These extracts were analyzed for b-RBP levels via colorimetric measurements after binding of streptavidin-peroxidase. Results are reported as a percentage of old to young mice. In calculating this percentage, paired (young and old) data was used for each experimental result; and this resulted in a much lower SEM for lung tissue than in the previous figure. Asterisk * indicates a statistically significant difference ($p < 0.05$) in the comparison of old and young accumulation values for a given tissue.

Accumulation of injected b-RBP in the same tissues of young and old mice was also analyzed at 4 h post-injection (p.i.). At this later time, the differences between young and old tissues were smaller than at half hour p.i. for both liver and kidney; for lung tissue, however, at even larger difference was observed at 4h. Figure 4-3 shows the change in the old/young ratios from 0.5 h to 4 h p.i., and is discussed further in Section 4.3.

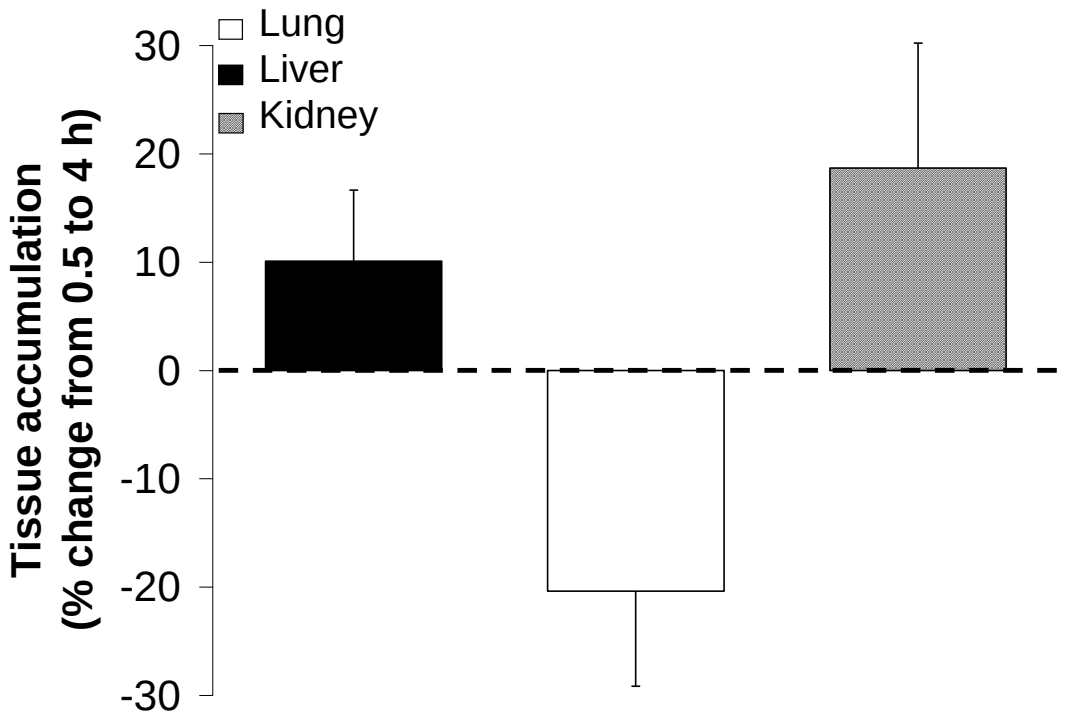

Figure 4-3 Relative change in accumulation of b-RBP between 30 minutes and 4 hours post-injection

Biotinylated RBP (b-RBP) was injected into young and old mice. At 30 min or 4 hours post-injection, tissue extracts were prepared (see Materials and Methods). These extracts were analyzed for b-RBP levels via colorimetric measurements after binding of streptavidin-peroxidase. Results are reported as the percentage change in O/Y ratios for the two time points.

4.2.2 Biotin-RBP binding to crude membrane preparations from tissues of young and old mice

In order to discuss possible age-related differences in RBP transport to cells and tissues, a related analysis of the total RBP binding capacity of the tissues or cells was required. Differences in accumulation, for example, may not be due to differences in the internalization process *per se*, but may be due instead to the relative abundance of membrane receptors (or to different RBP degradation rates) in young an old tissues.

Figure 4-4 shows a comparison of total b-RBP membrane binding, ratio of old to young values, for each of the tissue samples tested: lung, liver, and kidney. The highest ratio was in the lung, while the lowest ratio was in the kidney tissue. The total binding capacities of old tissues were all above 100%, which indicates that old tissues did not have decreased binding capacity. For both lung and kidney, the aging related increases (about 10-30%) in b-RBP binding capacity were statistically significant relative to the respective young tissues.

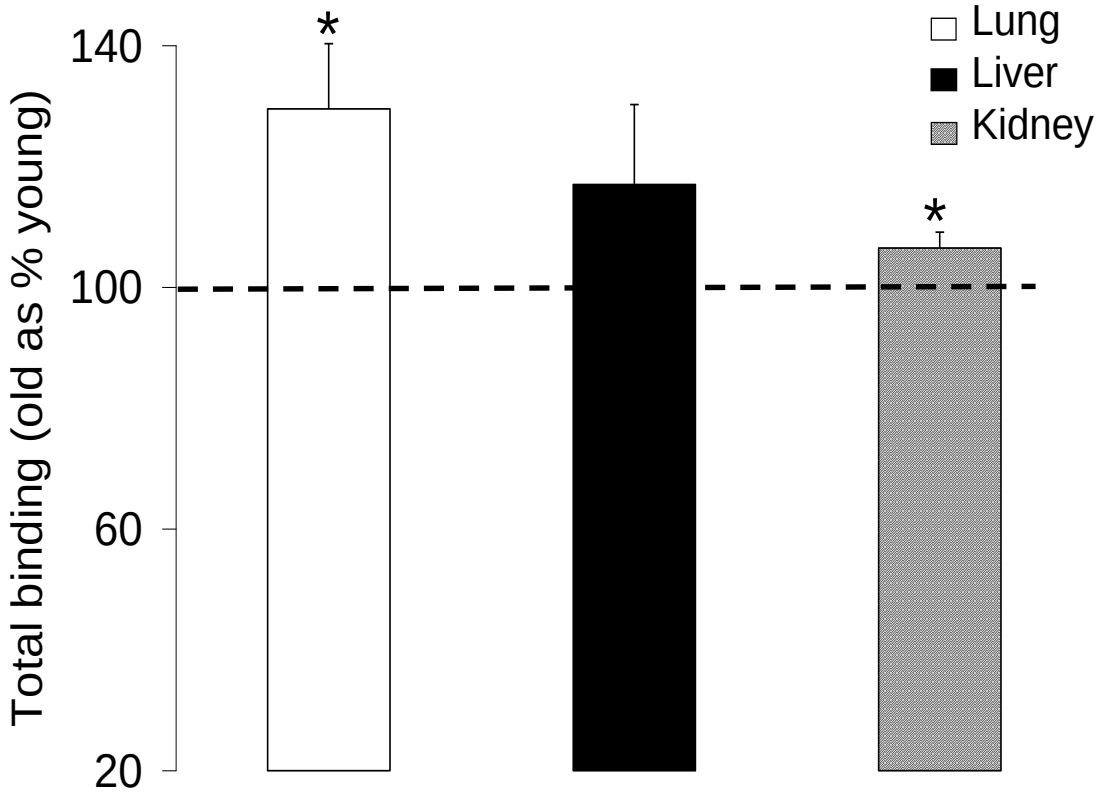

Figure 4-4 Relative total b-RBP binding to membranes prepared from young and old mouse tissues.

Tissue membrane extracts were prepared from lung, liver, and kidney samples using a detergent extraction procedure. The extracts were incubated with biotinylated RBP (b-RBP). The membrane extracts were analyzed for b-RBP binding via colorimetric measurement after binding of streptavidin-peroxidase. Results are reported as a b-RBP membrane binding percentage of old to young mice. Asterisk * indicates a statistically significant difference in the comparison of old and young binding values for a given tissue.

4.2.3 Comparative effects of aging on possible endocytic trafficking efficiency

By correcting total half-hour accumulation (Figure 4-2) for differences in total binding (Figure 4-4), an estimate of relative endocytic efficiency (possible rates of uptake) may be obtained. This estimate requires the assumption, however, that possible differences in other biochemical events (e.g., b-RBP degradation) within the cells over the half-hour period are negligible. Figure 4-5 shows a remarkably similar estimate, about 75%, of possible endocytic efficiency in old versus young tissues. This, in turn, suggests a 25% decrease endocytic efficiency in these tissues. As discussed further below, this value is within the range of age-related endocytic disruptions suggested by other studies which involved the use of various other ligands.

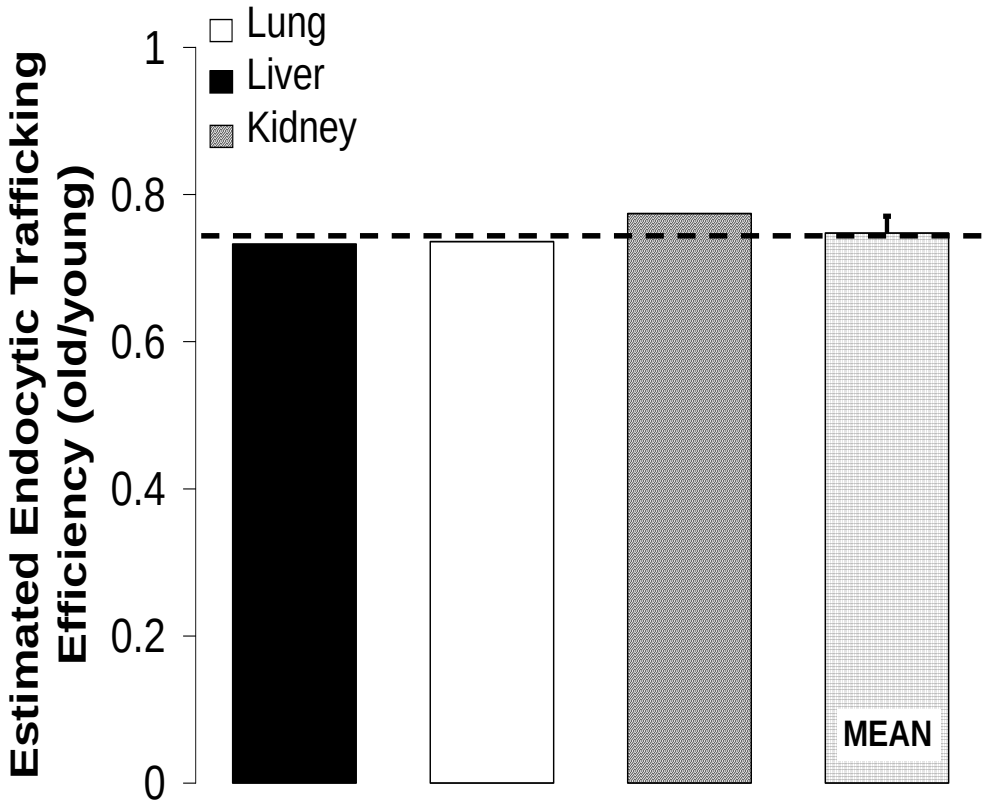

Figure 4-5 Estimation of endocytic trafficking efficiency in young and old tissues.

This figure represents a ratio of the values reported in Figure 4-2 to those reported in Figure 4-4. The mean is shown for all three tissues combined.

4.3 Discussion

Crude membranes from the three tissues examined—lung, liver, and kidney—prepared from old mice had more total RBP binding capacity than those from young mice; but the tissues from old mice accumulated less of the injected b-RBP 30 minutes post-injection. The 30 minutes accumulation is likely to be strongly affected by endocytic efficiency, but also may reflect recycling efficiency and other possible biochemical events such as degradation. To get an idea of true endocytic efficiency, future experiments should be performed on cells (e.g., primary cells from young and old animals) in culture where the early endocytic processes may be monitored at shorter accumulation time points, e.g., 5-10 minutes; at such time points other possible confounding events such as degradation and recycling are negligible.

In the experiments, it was also assumed that the method of euthanasia, carbon dioxide asphyxiation, has a negligible effect on the tissue b-RBP accumulation values. Evidence has not been found for any such effects in the literature, nor for any selective effects in young vs. old animals that could influence accumulation values to the extent observed. To be certain that the short period of carbon dioxide stress did not influence these results, however, future experiments could be performed with a different, rapid method of euthanasia such as decapitation.

The simplest explanation for these results is that of a similar and decreased efficiency of endocytosis in the three tissues from old animals relative to young, a loss of about 25% in such efficiency (e.g., 25% lower rate of uptake). Table 4-1 provides a comparison of aging-related decreases in endocytosis in various tissues and cells. Most studies suggest that aging results in an endocytic deficiency of 20-50% for the RME of various ligands, and the current values fall within this range. All of these studies, including those presented in this thesis, are based on rodents—mice or rats—as experimental models. The most intensively studied of the aging effects are those upon liver cells, Kupfer cells, liver sinusoidal endothelial cells (LSECs), and stellate cells (Le Couteur et al., 2008). The average (± SD) age-related deficit for six of the reported studies on liver cells was calculated to be 40.3 ± 12.3 percent, a value comparable to the 27 percent deficit that was observed in this study.

At times later than 30 min post-injection, the interpretation of accumulation signals becomes more complicated because of the increased importance of biochemical processes other than endocytosis, e.g., possible recycling and degradation. If aging affects endocytic transport much more than it affects other biochemical processes, then the accumulation levels at later time points should differ less between young and old tissues. As shown in Figure 4-3 this was indeed observed for liver and kidney tissues; lung, however, did not follow this trend and exhibited a greater difference at 4h p.i. vs. 30 min p.i.. This results suggests that some different effects of aging on

RBP transport/degradation occur in lung tissue relative to liver or kidney. Some possibilities for this include a higher rate of RBP proteolysis, or of RBP recycling back to the circulation, in lung relative to the other two tissues analyzed.

Table 4-1 A comparison of possible effects of aging on endocytic efficiency in rodent tissues and cells.

Cells/Tissues and Organisms	**Age comparisons** (in months unless noted otherwise)	**Age-related decrease in endocytosis** (ligand)
Kupfer cells (rat liver)	*nd*	35% (colloidal carbon)[1]
	6-12 vs. 22-36	23-58% (denatured albumin)[2]
Sinusoidal endothelial cells (LSEC; rat liver)	6-12 vs. 22-28	53-80% (azo-labelled albumins)[3]
Sinusoidal endothelial cells (LSEC; mouse liver)	3 vs. 27	~50% (modified albumins)[4]
Liver tissue extract (mouse)	1-2 vs. 28	27% (b-RBP)[5]
Hepatocytes (rat liver)	2 vs. 24	36% (Cu, Zn superoxide dismutase)[6]

Lung tissue extract (mouse)	1-2 vs. 28	26% (b-RBP)[5]
Kidney tissue extract (mouse)	1-2 vs. 28	23% (b-RBP)[5]
Brush border membranes (rat kidney)	3 vs. 24	30% (albumin)[8]
B cells (mouse Peyer's patches)	*nd*	~50% (immunoglubulins against IgM, IgG, IgA)[7]
Neurons (rat hippocampal & cortical)	*nd* (7-10 vs. 23-30 cell divisions)	25-52% (transferrin)[9]

[1]Videla et al., 2001; [2]Caperna and Garvey 1982, Heil et al. 1984, Brouwer et al. 1985; [3]Caperna and Garvey 1982, Heil et al. 1984; [4]Ito et al. 2007; [5]Current study, estimated endocytic efficiency; [6]Dini et al. 16; [7]Haq and Szewczuk 1992; [8]Cessac et al. 1993, estimated endocytic efficiency; [9]Blanpied et al. 2003; *nd* means 'no data'.

CHAPTER 5: MODULATION OF NUTRIENT TRANSPORT: SOME PHARMACOLOGICAL AND OTHER PHYSIOLOGICAL ASPECTS

5.1 RBP and vitamin A, and some other vitamins-vitamin transporters: an overview

Modulation of nutrient transport has many physiological and potential pathological consequences (references below and in Section 5.2.1). For example, transport of the vitamin biotin into cells has been related to chromatin remodelling and epigenetic effects (Zempleni et al., 2009) that could have a wide range of physio-pathological consequences. In the case of RBP and vitamin A delivery, it is likely that decreased transport or decreased cellular availability of retinoids will also influence genetic and epigenetic mechanisms; this has been suggested by studies of mutations in STRA6, and mutations in intracellular retinoid transporters and of enzymes that participate in retinoid metabolism. For example, a blockage in the supply of RA to the

nucleus in human epithelial cells can result in epigenetic (heritable, chromatin repressive) changes, likely based on DNA methylation patterns (Corlazzoli et al., 2009). Modulation of retinoid transport may also influence progression of Alzheimer's disease (Goodman and Pardee, 2003), and may underlie the toxic effects of some environmental pollutants (Novak et al., 2008). Moreover, a novel therapeutic strategy has been reported for liver cirrhosis that is based on RBP and vitamin A liposomes, and may involve receptor-mediated endocytosis of RBP (Sato et al., 2008).

Although, modulation of retinoid transport is not an immediate goal of the present thesis work, an endocytic transport assay has been developed that may help identify factors the influence endocytic transport in general and, more specifically, the receptor-mediated uptake of RBP. As detailed in this unit, the assay has been developed and tested using the well-established b-Tf-TfR ligand-receptor experimental system. In future work, it may be applied to other ligand-receptor systems including RBP-RBPR.

5.2 Towards identifying modulators of nutrient transport: a screening assay based on transferrin endocytosis

5.2.1 Introduction

Although not yet extensively explored as pharmacological targets, cellular internalization pathways are being considered more frequently in the context of pathological and therapeutic processes (Baranova et al., 2005; Eisinger and Schulz, 2005; Abulrob et al., 2005; Liu et al., 2005; Nathoo et al., 2003; Tanaka et al., 2004; Schonberger et al., 2003; Cam et al., 2005; Kenakin, 2005; Baskys et al., 2005; Hoppe et al., 2004; Guo et al., 2007; Caetano et al., 2008; Zhuang et al., 2009).

The main objective in relation to this part of the thesis was to develop a rapid and sensitive experimental strategy for quantifying endocytosis, while avoiding the use of radioactive isotopes. This could be potentially achieved through the use of a ligand-peroxidase conjugate (similar to that used in recycling experiments). The initial hypothesis was to develop such an experimental system, and that it would have a sufficiently high signal to noise ratio to be useful for screening potential endocytic transport modulators. The non-radioactive, rapid method could be useful for screening synthetic drugs or natural products such as phytochemicals, that stimulate or inhibit RME/CME.

Morevover, this assay could be adapted to some of the other studies in our laboratory, for example, related to the pathological mechanisms of protein

aggregates and amyloid fibrils; one of the hypotheses with such studies is that these aggregates disrupt endocytic transport mechanisms and this effect contributes to their pathological effects in the body. Aggregated forms of proteins currently under study (e.g., TTR, Section 1.2.1) could be tested in this transport assay and their possible effects on endocytic transport could be quantified.

5.2.2 Results and discussion

5.2.2.1 Validation of the assay

Transferrin has been a model ligand for many investigations of RME and endocytic trafficking, e.g., (Rapaport et al., 2006; Davis et al., 1987; Wiley and Kaplan, 1984; Thomas-Crusells et al., 2003; Ajioka and Kaplan, 1986; Ward et al., 1982; Hopkins and Trowbridge, 1983). A biotinlyated transferrin-streptavidin peroxidase (ESa-bTf) complex was used to quantify endocytosis of transferrin. A schematic of the ESa-bTf ligand placed within the known steps of Tf RME is shown in Figure 5-1, and provides an overview of the assay.

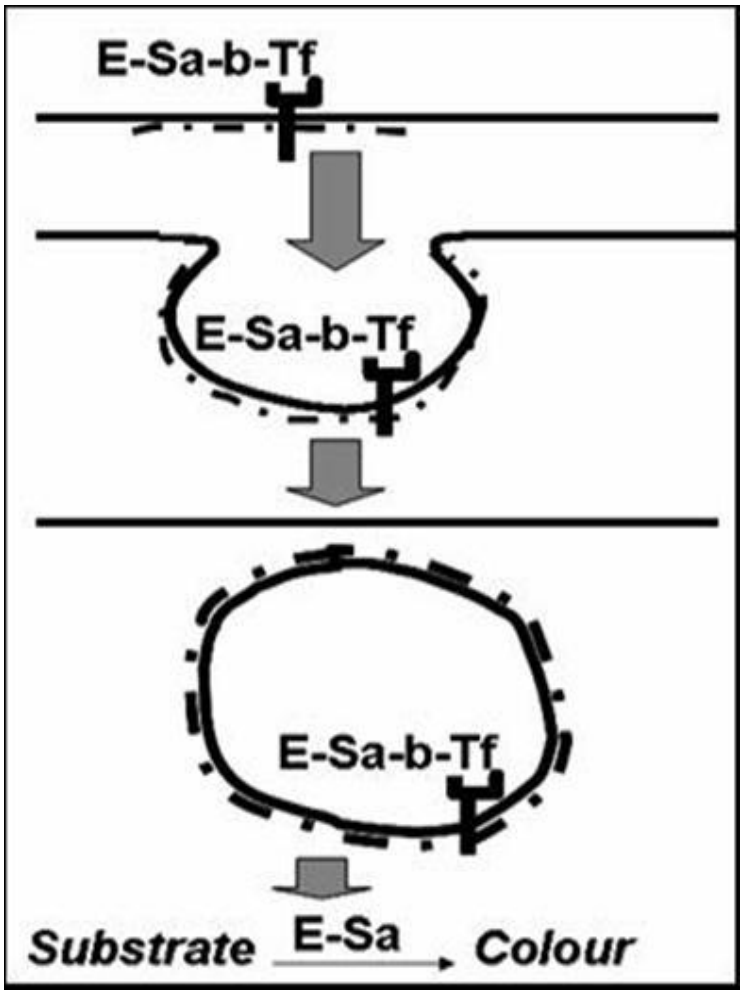

Figure 5-1 Schematic of the assay in relation to the known steps of clathrin mediated endocytosis.

In this example, the conjugate of biotinylated (b) ligand (Tf, transferrin used as a model ligand) and streptavidin (Sa) peroxidase (E) is shown to be internalized by a receptor for the ligand located in clathin-coated pits. The presence of potential modulators may affect the amount of Tf that accumulates within the membrane-enclosed vesicle, the final step shown above. After removal of all labelled Tf that has not been 'captured' within a membrane-bound compartment, the cells are lysed with detergent and the enzymatic activity is quantified through a color reaction.

As shown in Figure 5-2, a relatively strong signal is observed for the uptake of the complete Tf conjugate (ESa-bTf, first bar). The low (control) signals obtained in assays with excess, unlabelled Tf (ESa-bTf + Tf, third bar) and with the ESa conjugate alone (ESa, second bar) indicate specificity of the Tf component of the complete conjugate. Moreover, excess, unlabelled Tf does not significantly affect the ESa-only signal (ESa + Tf, fourth bar), an indication that it does not participate in non-specific competition. These three relatively weak control signals (2^{nd}-4^{th} bars) likely represent mainly non-specific, fluid-phase uptake, i.e., not the expected RME process of the complete Tf conjugate. It is possible that with a greater excess of Tf, the ESa-bTf + Tf control signal (3^{rd} bar) could be decreased further.

The assay has a signal-noise (S/N) ratio in the range of about 2-4 (Figure 5-2, difference between 1^{st} and other three bars). This is a value that should be sufficient to identify strong stimulators or inhibitors of ligand accumulation in the screens. Assays with similar S/N ratios have been used for screening membrane-related activities or other analytical methods (Fukushima et al., 2001; Xie et al., 2007). An added advantage of the assay is that it can be performed in cells growing on 96-well plates; thus, different potential modulators at various concentrations may be tested in a single-plate assay.

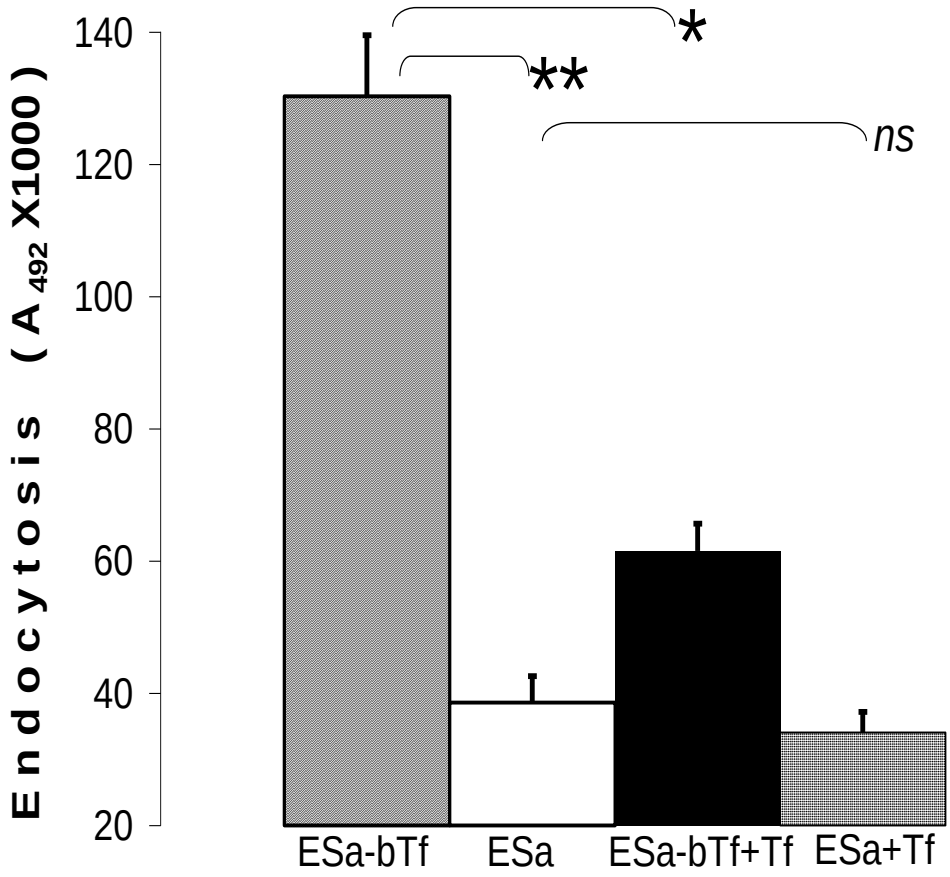

Figure 5-2 Quantification of transferrin endocytosis using the novel ligand-enzyme conjugate assay.

Validation of assay performed with A431 cells with the indicated conjugates (see Experimental Procedures). The raw spectrophotometric data (absorbance at 492 nm) was used for the graph. Competition of ESa-bTf endocytosis was performed with an excess of unlabelled holo-human Tf. Lines with asterisks or "ns" (non-significant) refer to statistical significance (by t-tests, see Experimental Procedures; *, p between 0.05 and 0.01; **, $p < 0.01$) of the indicated comparisons. Definition of symbols: E, peroxidase; Sa, streptavidin; b, biotin; Tf, transferrin.

5.2.2.2 Testing of the assay in screening for potential endocytic modulators

A major application of the proposed assay and one of particular interest to our research group, is screening of phytochemicals and other dietary/natural products, or synthetic drugs, for their ability to modulate RME and endocytic trafficking. Strong inhibitors or stimulators could then be further analyzed, e.g., in assays for other pathological and therapeutic processes. An extensive screening is beyond the scope of the current thesis; but some results from the screens are presented here to illustrate potential applications.

As an example of the use of the assay, three phytochemical extracts were analyzed (see Materials and Methods), from *Ginkgo Biloba*, *Vaccinium myrtillus*, and *Capsicum annuum*. These plants have been widely investigated for antioxidant and other potential biomedical effects (Oliveira et al., 2009; Persson et al., 2009; Shi et al., 2009; Yao and Vieira 2007; Takano et al., 2007; Oboh and Rocha, 2008). As shown inTable 5-1, treatment of cells with the Ginkgo, but not the Vaccinium, extract resulted in a significantly enhanced cellular accumulation of b-Tf. The Capsicum extract, on the other hand, resulted in an inhibition of b-Tf endocytosis. These results represent a basis for future work:

(i) to verify a specific effect on endocytosis, the effect of the same treatment on cell membrane b-Tf binding capacity should be performed;

(ii) if there is a specific effect on endocytic efficiency, the extracts could then be subjected to fractionation, e.g. by HPLC, with the goal of identifying the active substance or the minimal combination of active components.

In the context of the latter future goal, a recent report indicates that a fraction of a *Cinnamomi cortex* plant extract could decrease clathrin-mediated endocytosis of the Tf receptor (Zhuang et al., 2009). Moreover, such inhibition of endocytic transport may account, at least in part, for the ability of this sub-fraction to reduce infectivity of a severe acute respiratory syndrome (SARS) virus.

It is also planned that in future work with the assay, *purified* phyto-chemicals (and other drugs) will also be screened for their effects on endocytic transport. Any such purified compounds identified would not require further purification (unlike the cases mentioned above) and could be directly studied in other cellular and whole-body physiological systems. Recently evidence in the current study shows an inhibitory effect of purified ascorbate (vitamin C) in the b-Tf endocytic transport assay (about 50% inhibition relative to control without ascorbate, $p < 0.05$). Others have reported that this vitamin can interfere with RME of Tf, and thereby may contribute to cell death (Kang et al., 2005; Carosio et al., 2005). At present, the reason for the ascorbate-mediate endocytic inhibition that is observed is not known, but one possibility is that it interacts with redox active metals in this experimental system and promotes oxidative stress (cf. Marcil et al., 2006; Chen et al., 2007; Chen et al., 2008).

Oxidative stress is known to inhibit endocytosis of Tf (Cheng and Vieira, 2006; Malomi et al., 1998). This example with ascorbate is presented to illustrate an important point related to the testing of purified compounds, even those that are considered to be primarily antioxidants such as ascorbate and polyphenolic phytochemicals: the inhibition may be due to secondary effects such as oxidative stress and not to a direct effect of the compound on one or more components of the endocytic machinery (or on the ligand-receptor interaction or surface expression levels of the receptor).

The possible stimulation of endocytosis by *Ginkgo biloba* seems intriguing and unique. If it is indeed verified that these extracts can stimulate endocytosis itself (and not of Tf receptor expression levels), it will be of interest to examine possible mechanisms such as increased levels of specific endocytic machinery components (cf. Blanpied et al., 2003) or increased activity of such components. A literature review did not provide any reports on phytochemicals or other drugs that document a stimulatory effect on the workings of the endocytic machinery (other than those that may increase the general level of available energy, ATP).

Table 5-1 Effects of cell treatments with some phytochemicals on endocytosis.

The newly developed endocytosis assay was applied to screening phytochemical extracts. Data from three of the aqueous extracts that were most extensively analyzed are presented. Each extraction was performed on 2 mg of dry plant material per ml solvent. In each case, the effect of the phytochemical extracts on b-Tf endocytosis is shown as percent control ± SEM. Control (100%) refers to a corresponding set of assays performed without phytochemicals. Asterisks indicate statistical significance determined by t-tests against control data, $p < 0.05$. Standardizations for two of the three extracts are given in the Materials and Methods. (A project is underway in the laboratory to standardize all the extracts in terms of total polyphenol content.)

	b-Tf endocytosis
Standardized *Gingko* extract (2 mg/ml aqueous)	137.7 ± 11.1*
Standardized *Vaccinium* extract (2 mg/ml aqueous)	113.7 ± 23.6
Non-standardized *Capsicum* extract (2 mg/ml aqueous)	54.2 ± 12.3*

It will be of interest to determine if some of the inhibitory or stimulatory phytochemicals (or other nutraceuticals/pharmaceuticals) have the same effects on other nutrient carriers such as RBP as they have upon Tf internalization. Thus, future experiments will also be directed towards modulation of RBP/vitamin A endocytic transport.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

The following are the main conclusions from the work done as part of this M.Sc. thesis project:

i. The A431 human epithelial cell line contains at least one specific RBP receptor of about 157 kDa.

ii. At least some, if not most, of internalized exogenous RBP can be recycled back out of the cell after endocytosis.

iii. Aging decreases the accumulation of circulatory RBP in some mouse tissues, especially in kidney tissues.

iv. A possible loss of about 25% in the endocytic transport efficiency of old mouse tissues (liver, kidney, lung) is suggested by the b-RBP accumulation studies at the earliest time point, 30 min, post injection.

v. A high-throughput cellular screen has been developed for identification of potential exogenous modulators of receptor-

mediated endocytosis and endocytic trafficking. At least one phytochemical extract (*Capsicum annuum*) has been identified by this assay for future sub-fractionation and identification of one or more possible endocytic modulatory compounds.

In terms of future directions of this research theme, as detailed in the main text, the following are considered to be the most immediate and important extensions:

a. to further characterize RBP recycling, for example, in terms of (i) the percentage of internalized RBP that is recycled and (ii) whether or not the internalized holo-RBP delivers its retinol cargo to the cell and recycles back out of the cell empty, in the apo-RBP form (as is the case with transferrin).

b. to identify and characterize possible RBP cellular signalling pathways, and determine if modulation of RBP RME influences such signalling.

c. to further establish age-related changes in the endocytic efficiency of RBP; future experiments should be performed on cells (e.g., primary cells from young and old animals) in culture where the early endocytic processes may be monitored at shorter accumulation time points, e.g., 5-10

minutes, that are not significantly affected by other events such as ligand proteolysis or recycling.

d. to determine whether endocytic modulators (e.g., phytochemicals, Section 5) have similar effects on different nutrient transport systems such as RBP-RBPR and Tf-TfR, or the clathrin-mediated endocytosis of other ligand-receptor systems.

e. to further characterize the RBP receptor that was identified in these studies; e.g., to obtain amino acid sequence and determine the type of protein (protein family) that it represents**.**

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