Effects of physiological, pharmacological, and toxic factors on cell transport

by

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Abstract

Endocytic transport is a fundamental cell function involved in nutrient assimilation, signal transduction, growth, and other cellular activities. Disruption of this transport has been associated with aging and some chronic diseases. There is growing interest in possible regulation of the endocytic machinery by physiological factors, and in pathological processes. In this project, I analyzed effects of (a) pro-oxidative factors such as ROS and amyloidogenic polypeptide aggregates, (b) physiological factors such as EGF and steroid hormones, and (c) select pharmacological or phytochemical compounds, on different endocytic pathways within a cell, and among different cell types. The results provide evidence that pro-oxidants can inhibit endocytic transport in different cell types; and the pathways can exhibit differences in sensitivity to a pro-oxidant. Select physiological and pharmacological factors can also stimulate or inhibit some of these transport pathways in different cell types. Further screening and testing of pharmacological modulators may identify possible therapeutic compounds.

Keywords: aging; cell transport; endocytosis; growth factors; oxidative stress; pharmacology; phytochemicals; steroid hormones

I dedicate this work and give special thanks to my family and many friends. I have been abundantly blessed with support throughout the process and I will always appreciate all that they have done. I also dedicate this work with a special feeling of gratitude to my mother, for her never-ending love and encouragement.

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Table of Contents

List of Tables

List of Figures

List of Acronyms

agTTR	Misfiled and aggregated TTR (pathological)
ASC	Ascorbate (vitamin C)
A431	A human epithelial (epidermoid) carcinoma cell line
atRA	All-trans retinoic acid
BNHS	Biotin N-hydroxysuccinimide ester
BSA	Bovine serum albumin
CME	Clathrin-mediated endocytosis
DBP	Vitamin D carrier protein
DMEM	Dulbecco's modified eagle medium
EB	Extraction buffer
EDTA	Ethylenediaminetetraacetic acid
E2	17 beta estradiol
EE	17 alpha ethinylestradiol
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ESa	Enzyme (E, peroxidase enzyme) complexed with streptavidin (Sa)
Esi	Esi-09, 3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-propionitrile
FBS	Fetal bovine serum
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRP	Horseradish peroxidase (plant origin)
Ig	Immunoglobulin (different types, e.g. IgG)
KSHM	buffer: 100 mM potassium acetate, 85 mM sucrose, 1 mM magnesium acetate, 20 mM HEPES-KOH, pH 7.4.
OPD	ortho-phenylene diamine
PBS	Phosphate-buffered saline
RME	Receptor-mediated endocytosis
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SFM	Serum free medium
SICM	Semi-intact cells and membranes
sNF	sNF94.3, a Schwannoma-like human cell line
sTTR	wild-type, soluble TTR (same as TTR, but different from agTTR)
TBS	Tris-buffered saline
Tf	Transferrin
TMPD	N,N,N',N'-tetramethy-1,4-phenylenediamine
TTR	Transthyretin (previously called pre-albumin)
VDBP	Vitamin D-binding protein

Executive Summary

Endocytic transport is a fundamental cell function that has implications for nutrient assimilation, signal transduction, growth, and other cell activities. Disruption of this transport has been associated with aging and may have implications for chronic diseases such as neurodegenerative disorders. Much of the work on endocytosis has been centered on characterization of the endocytic machinery. There is growing interest to examine how endocytic pathways are influenced by a wide range of physiological factors, and in pathological processes.

The main **objective** of the current thesis project was to examine possible modulation of this transport function by pathological, physiological, and pharmacological factors: pro-oxidants such as reactive oxygen species (ROS), redox-active metals, and amyloidogenic protein aggregates; epidermal growth factor (EGF) and steroid hormones; and select synthetic pharmacological and natural phytochemical compounds.

The main **methods** involved treating the cells with the above factors followed by transport assays using polypeptide ligands that represent different endocytic pathways, e.g., clathrin-mediated as well as non-specific uptake pathways. Controls included transport assays performed in the absence of the physiological or toxic factors, as well as pre-treatment versus acute treatment with the factors.

The **results** provided evidence that **(a)** different pro-oxidants (e.g., H_2O_2, iron-ascorbate) can inhibit endocytic transport in different cell types; and these pathways can exhibit differences in their sensitivity to the same pro-oxidant. Relative to a human epidermoid carcinoma cell line, murine primary liver cells were found to be about 20% more resistant to the inhibitory effects of 0.5 mM hydrogen peroxide ($p < 0.05$). **(b)** Physiological factors such as epidermal growth factor (EGF) can stimulate or inhibit different endocytic pathways in different cell types. A 30 min pre-treatment with 10 nM EGF could inhibit the clathrin-mediated endocytic (CME) pathway of Tf, but not the non-CME pathway of a calciferol-carrier protein, VDBP (about 15% inhibition for Tf relative to untreated controls, $p < 0.05$). **(c)** Pharmacological modulation of endocytic transport is possible, and may have applications in the therapy of some diseases. Among the compounds tested, a synthetic inhibitor of EPAC (exchange protein directly activated by cAMP), but not the antioxidant phytochemical apocynin, could inhibit the Tf endocytic pathway (about 25% inhibition at 20 μM, $p<0.05$ relative to controls).

In **conclusion**, novel regulatory factors and control mechanisms for endocytic transport have been identified, and partly characterized, in this M.Sc. thesis project.

Chapter 1. BACKGROUND INFORMATION, MAIN OBJECTIVES, AND HYPOTHESES

1.1. Overview of project and objectives

Endocytic transport is an essential function of cells. Such transport is involved in nutrient assimilation, and affects cellular signal transduction, growth, death, and differentiation. Disruptions of endocytosis are associated with aging (Blanpied et al., 2003; other references in Table 3.2), and likely contribute to chronic diseases including some cardiovascular diseases, cancers, diabetes and other metabolic diseases, kidney diseases, and neurodegenerative disorders (Nixon et al., 2005; Gauci et al., 2013; Christianson et al., 2013; Pataki et al., 1992; Dahmane et al., 1996; Tossibou et al., 2010; Tortorella and Karagiannis 2014; Fowlkes et al., 2011; Jo et al., 2014; Marcello et al., 2013; Robertson 2009; Saito et al., 2010; Svistounov et al., 2013; Williams and Palmer 2014). In many infectious diseases, the pathogen (virus, bacterium, parasite; reviewed by Vega and Cossart, 2006; Long et al., 2006) must be endocytosed for disease progression; interference with such transport may prevent or decrease severity of the infection. Moreover, delivery of therapeutic agents can be based upon well-characterized endocytic transport systems such as that of the iron carrier protein transferrin (Tf) and its cell surface receptor (Tortorella and Karagiannis 2014).

Much of the research in this field has been focused on the characterization of the endocytic machinery, but there is growing interest in studying more of the physiological and pathological processes that modulate endocytic transport. In this thesis project, some physiological and toxic factors have been examined for their effects on specific (e.g., clathrin-mediated, CME) and non-specific endocytic pathways. Factors of interest included pro-oxidants, phytochemical antioxidants, pathological (amyloidogenic) protein aggregates, as well as physiological regulators such as epidermal growth factor and steroid hormones.

The objectives of my project were to characterize and better understand: **(1)** the effects of selected pro-oxidants and antioxidants on endocytic pathways; **(2)** potential effects of other toxic factors such as membrane disruptors, and of aging, on endocytosis; and **(3)** potential modulation of endocytosis by physiological factors such as epidermal growth factor (EGF). A list of specific hypotheses that were tested is given at the end of this Chapter.

1.2. Background

1.2.1. Endocytic transport and its relation to physiology and pathology

Different pathways into the cell

Endocytosis is typically considered as an energy-utilizing process which results in the uptake of substances (e.g., polypeptides or nutrients) from the

extracellular environment. It is a fundamental transport mechanism used by all cells of the body, and has the ability to control the cell's relationship with the extracellular environment as well as intracellular signaling (Sigismund et al., 2012). Endocytic transport can also participate in the regulation of cell-surface receptor expression, cell adhesion and migration, cholesterol transport, cell polarity, pathogen entry, synaptic transmission, antigen presentation, cell growth and differentiation, and drug delivery.

Large substances or polar molecules cannot readily pass through the hydrophobic cell membrane; but they can enter the cell through different endocytic pathways. Historically, the four best characterized endocytic pathways were clathrin-mediated endocytosis, caveolar endocytosis, macropinocytosis, and phagocytosis. These pathways are included in **Figure 1.1,** a schematic representation of the main categories of endocytosis.

Figure 1.1. Three main categories of endocytic transport.

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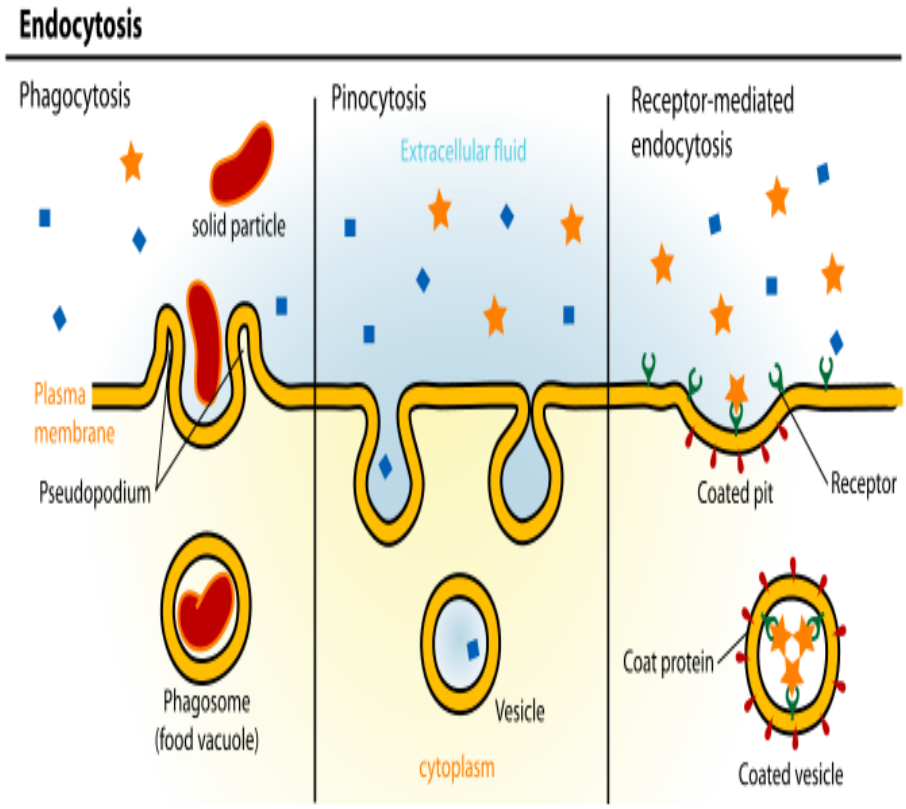
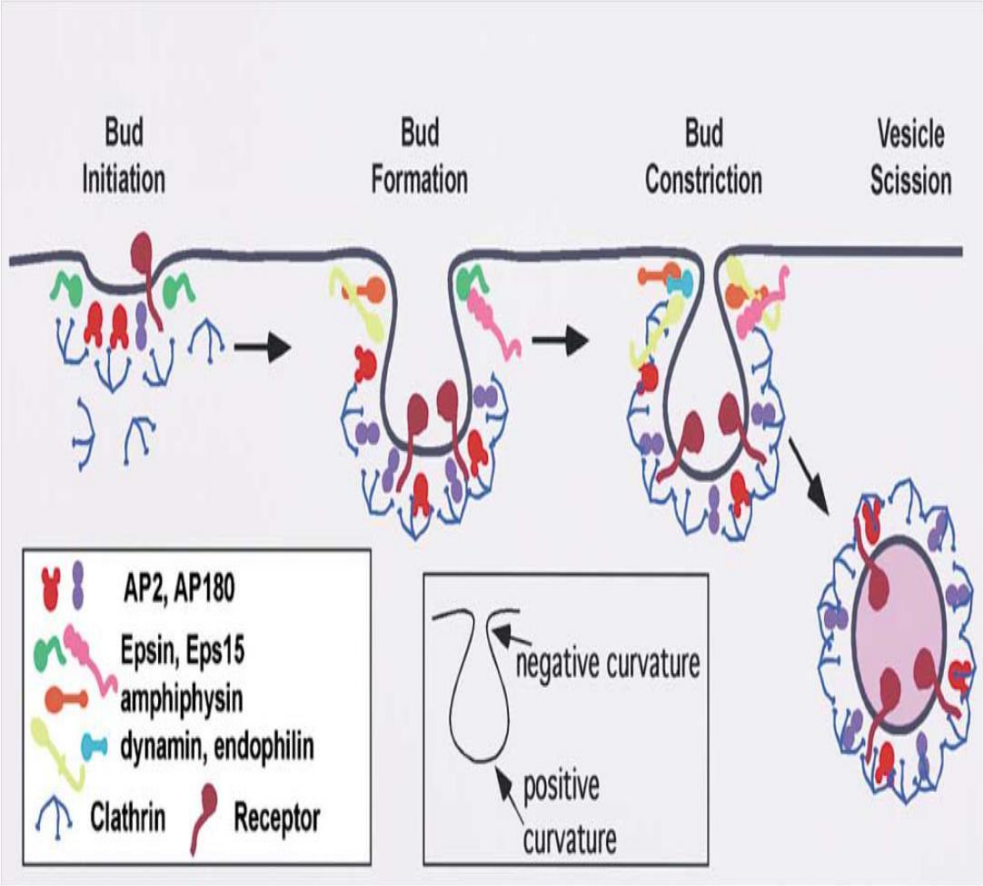


Clathrin-mediated endocytosis (CME) occurs through clathrin-coated vesicles, after budding of clathrin-coated membrane pits. CME occurs ubiquitously throughout the body, and is the best characterized pathway. In my studies I used the CME of Tf (transferrin) as a standard transport assay for testing effects of possible modulators. **Figure 1.2** shows a schematic of some of the endocytic machinery involved in CME.

Another type of intracellular membrane coating is represented by caveolae. The coat protein, caveolin, is a small cholesterol-binding protein. Caveolae are abundant in smooth muscle cells, lung tissues, adipocytes, and endothelial cells (McMahon and Boucrot, 2011). Many extracellular molecules can be endocytosed after binding to cell membrane receptors through receptor-mediated endocytosis (RME). These receptors can be present in clathrin-coated or caveolin-coated or other membrane regions. Classical examples of RME via CME include the lipoprotein LDL and, as mentioned, the iron carrier protein Tf. Their endocytic pathways are well characterized, and they often serve as standards in endocytosis studies. Once internalized, components of endocytic vesicles can either be recycled back out of the cell or sorted for other functions or for degradation (McMahon and Boucrot, 2011).

Figure 1.2. Some of the main steps and components of the CME machinery

From Hurley et al. (2012). The process begins with bud initiation and assembly of the clathrin coat through adapter proteins that link it to the membrane. Eventually, a clathrin-coated vesicle is formed in the cell. The vesicle can then be uncoated and can fuse with early endosome organelles.



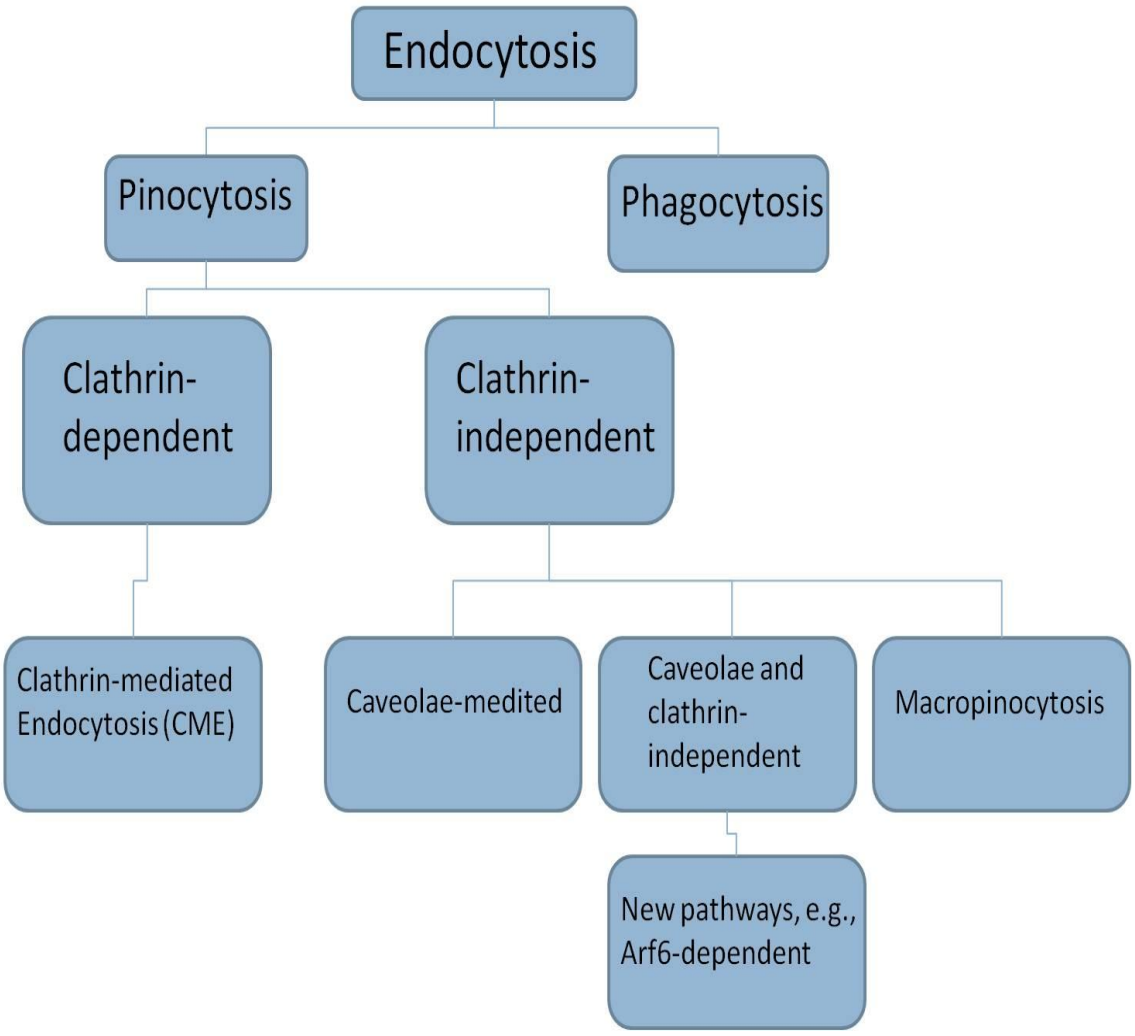
Macropinocytosis can occur in non-coated regions of the plasma membrane. As with other endocytic processes, it involves invagination of the cell membrane to form intracellular vesicles. This non-specific transport mechanism can engulf large volumes of extracellular fluid and solutes present in the fluid. The vesicles travel deeper into the cytosol and later fuse with other membrane-bound compartments such as endosomes and lysosomes.

Phagocytosis is the process in which large particulate matter such as cell debris, micro-organisms, and apoptotic cells is internalized. Infectious agents such as bacteria can be phagocytosed, and this process contributes to their infectivity.

More recently, some have suggested that the previous classification of endocytic pathways is inadequate, and a better classification system is needed which can account for additional, newly identified but poorly understood pathways. Because of this increasing complexity, a more suitable approach may be to classify the endocytic mechanisms based on clathrin-dependence with various subtypes of clathrin-dependent and clathrin-independent pathways; an example is shown in **Figure 1.3**.

Figure 1.3. A classification system for different endocytic pathways

Based on a similar figure by Sahay et al. (2011).



1.2.2. Oxidative stress and its relation to endocytosis

Oxidative Stress

Oxidative stress occurs regularly throughout the cells and tissues of the body and increases during aging (Lee et al., 2012; Rodriguez-Biez et al., 2014, and additional references in Discussion). During normal metabolic function, reactive oxygen species (ROS) form as by-products of energy metabolism. If produced in excess or inefficiently eliminated, ROS such as hydroxyl radicals and hydrogen peroxide can damage many components of cells and tissues including proteins, lipids, and DNA (Dickinson 2011). Different antioxidant mechanisms can protect the body's cells and tissues through the moderation of ROS production or by directly neutralizing the reactive species. Some reactive species play a role in cell-messenger cascades in reduction-oxidation (redox) cell signaling (Lee et al., 2012; Kano et al., 2011), and are part of normal physiology. Reactive chemical species are also normally utilized by the immune system as a means of protecting the body against pathogens; they can damage and destroy some of these infectious agents.

From a chemical point of view, oxidative stress is a result of an imbalance between the production of reactive oxidizing species on one side, and antioxidant capability and damage repair on the other. Both can be the result of numerous

conditions: e.g., metabolic disturbances from diseases, nutritional deficiencies or excesses, accumulation of toxins from the environment, the aging processes, etc. ROS include the following: superoxide radical, hydroxyl radical, hydrogen peroxide, hypochlorous acid, lipid peroxyl radicals. In biomedical studies of oxidative stress, hydrogen peroxide is commonly tested because of its relative stability; and elevated levels have been implicated in pathological processes such as those that involve neuroinflammation and neurodegeneration (Lee et al., 2012; Fang et al., 2012; Kano et al., 2011).

Oxidative stress and the resulting oxidative damage is thought to be fundamentally involved in the development of age-related neurological diseases, e.g., Parkinson's Disease, Alzheimer's Disease, Lou Gehrig's Disease, Huntington's Disease, and the development of cardiovascular diseases, e.g., atherosclerosis, myocardial infarction, heart failure, and the development of some types of cancer. Recent studies have suggested that oxidative stress plays a role in some developmental disorders such as autism, Chronic Fatigue Syndrome, and genetic disorders such as Sickle Cell Disease, and Fragile X Syndrome (Lee et al., 2012; Kano et al., 2011; Dickinson et al., 2011).

The homeostatic balance between production and removal of ROS is essential for overall health. Recent studies have focused on the benefits of naturally occurring antioxidants to overall health and longevity. It is debatable as to whether antioxidant treatment is beneficial in preventing disease; the evidence is not very strong. For example, smokers who take high doses of synthetic beta-

carotene had an increased risk of lung cancers compared to smokers who took the placebo (Miller et al., 2005; Ames et al., 1995). Vitamin E supplementation also appears to have mixed efficacy on reducing the risk of Alzheimer's and cardiovascular diseases (Lee et al., 2012; Kano et al., 2011; Miller et al., 2005). A significant portion of the mixed results may be due to the difference in effectiveness of whole foods compared with isolated compounds. Dietary sources contain a larger spectrum of antioxidants and other compounds that can interact and work synergistically with the compound of interest. As a result, the isolated compound can behave differently in clinical tests.

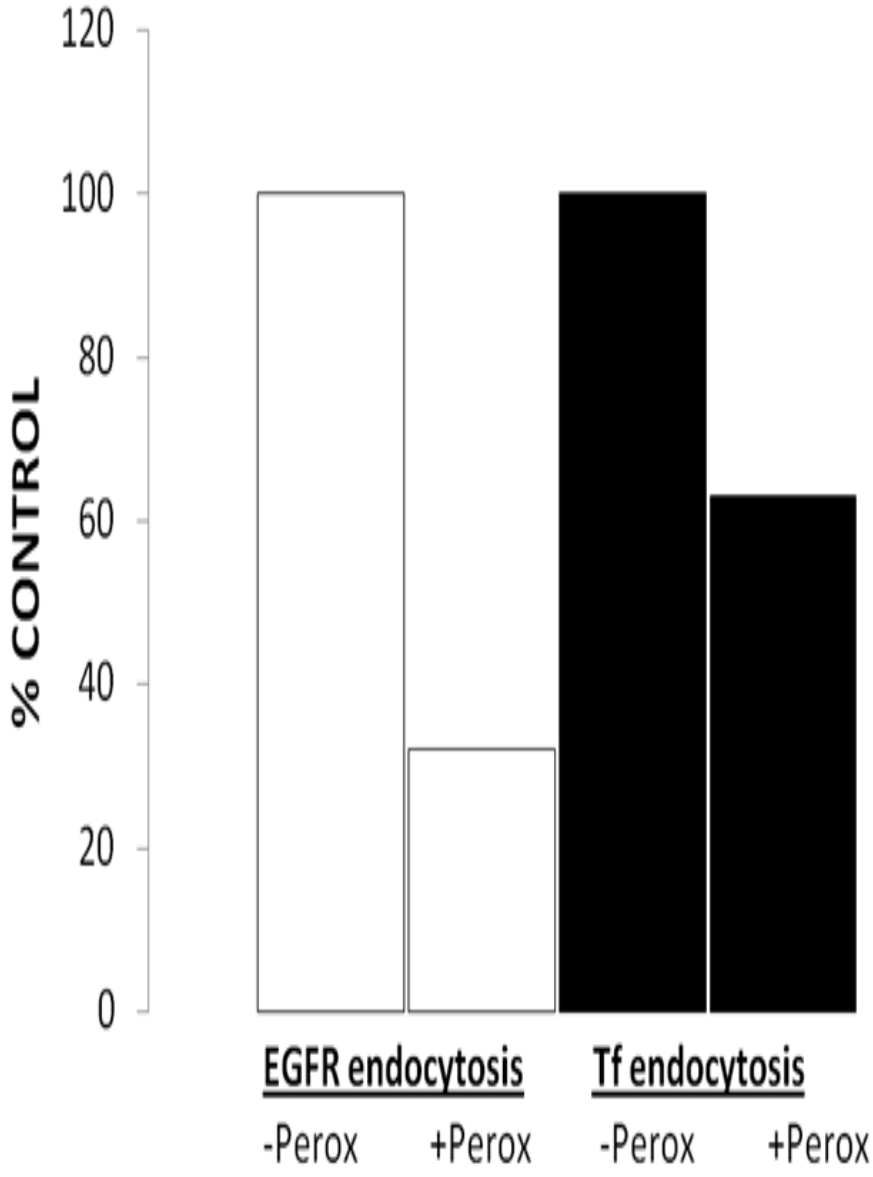
Inhibition of Endocytosis by Pro-Oxidative Factors

Previous studies have shown that oxidative stress can have an effect on endocytosis. Kano et al. (2011) as well as Cheng and Vieira (2006) reported that treatment of human cell lines with H_2O_2 impaired internalization of the EGF receptor (EGFR) and of transferrin, two clathrin-mediated events; **Figure 1.4** shows some of the results obtained in these two studies. Internalization of insulin can also be inhibited by oxidative stress (Bertelsen et al., 2001). Impairment by pro-oxidants is not limited to mammalian cells: Pereira et al (2012) reported that H_2O_2 caused a concentration-dependent inhibition of endocytosis in yeast, an effect that was reversible upon removal of peroxide.

Lower endocytic efficiency has been associated with aging, e.g., comparisons among young and old cells (Blanpied et al., 2003). This decreased transport efficiency may be, at least partly, a result of high levels of oxidative stress that are also associated with aging (e.g., Gan et al., 2012). Some experiments were performed as part of this thesis project to examine such a relation between aging and oxidation. Section 3.1.4 contains additional examples of possible effects of aging on endocytosis.

Figure 1.4. Graphic illustration of the main findings previously reported by Kano et al. (2011), and Cheng et al. (2006).

Inhibition of clathrin-mediated endocytosis by hydrogen peroxide, 0.5 mM (white) and 0.1 mM (black), 5 min endocytosis of EGFR in HeLa (white) and of Tf in A431 (black) human cell lines. This figure is intended to show the approximate magnitude of the peroxide-induced inhibition. Complete statistical analyses and experimental procedures and can be found in the respective reports.



Cells can utilize different mechanisms and pathways of endocytosis, and these may be differentially affected by pro-oxidants. Currently, there are many questions regarding such potential differential effects. In the current project, I investigated this further by using ligands that represent different endocytic pathways.

In addition to examining the effects of oxidative stress on a membrane function—endocytosis—it is also important to consider its effects on membrane structure and cell surface receptor expression levels. A decrease in endocytic function may not necessarily be the result of decreased endocytic machinery function, but may involve membrane structural changes that, for example, decrease the number of available binding sites. The structural integrity of the membrane may also influence the ligand binding affinity or membrane movement (fluidity) of the receptors.

1.2.3. Modulation of endocytosis by physiological factors

Hormones, growth factors, and nutrients all play a role in maintaining health and homeostasis. Some of these physiological factors can affect the rate of endocytosis, thereby affecting nutrient uptake rates, cellular signaling, and growth. Sex steroids, for example, have been reported to affect endocytosis

(Yang et al., 2005; Bonacorsi et al., 2007), but the extent of their effects among different cell types, and the pathways affected, remain poorly understood. The growth factor EGF (epidermal growth factor) is another physiological regulator of interest in terms of its potential effects on endocytosis. To date, there are indications that EGF can influence signalling components associated with endocytic machinery or compartments. However, in terms of possible shorter-term direct effects of EGF on endocytic pathways such as CME of Tf, I have found few reports (Section 4.2.2; Sandvig and vanDeurs, 1990). I investigated possible regulation by these physiological factors using ligands that represent some of the endocytic transport pathways, e.g., CME of Tf and and non-CME pathways.

1.2.4. Modulation of endocytosis by phytochemicals and pharmacological factors

Phytochemicals are plant chemicals. This term is commonly applied to compounds found in plant-based foods and plant-derived dietary supplements that may have effects on human health. Phytochemicals can modulate the function of animal cells through a variety of different molecular mechanisms such as effects on signal transduction and apoptosis, epigenetic control, and transport activities (Fulda and Debatin, 2006; Rinwa et al., 2013; Thakur et al., 2014;

Arango et al., 2013; Rubio et al., 2014; Tan et al., 2013). In terms of the latter activity, there is evidence for modulation of endocytic transport by some phytochemical compounds. For example, genistein inhibits signalling through receptor-tyrosine kinases (RTKs) and can inhibit non-clathrin endocytic pathways; it has been used as an inhibitor of caveolar endocytosis (Rejman et al., 2005; Long 2006)

Because endocytic transport is a fundamental cell function with physiological and pathological consequences, there is the possibility that modulation of this basic function through pharmacological agents may be useful in preventing or controlling some diseases. For example, as mentioned above, many infectious diseases require endocytosis of the microbe for disease development, and if such uptake can be decreased or blocked, infection may be prevented or moderated (Long et al., 2006; Vega and Cossart, 2006; Huang et al., 2012). A compound recently identified by a collaborator (3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chlorophenyl)-hydrazono] -3-oxo-propionitrile) inhibits the EPAC enzyme (exchange protein directly activated by cAMP), and also acts as a pharmacological compound that can protect experimental animals from fatal rickettsiosis (Dong et al., 2013, and additional references in Discussion). Because it prevents invasion of animal cells by these bacteria, it likely affects some form of endocytic transport. I have tested this compound in cell transport assays to determine if it affects internalization of marker proteins by cells, e.g., the CME of Tf.

1.3. Hypotheses

The following are hypotheses of my thesis project. Hypotheses 1-4 relate to the effects of oxidative stress. Hypothesis 5 represents an extension of previous studies that have examined estrogens and epidermal growth factor (EGF).

1. Pro-oxidants inhibit endocytosis, and the extent of such inhibition differs with different pro-oxidants.

2. Multiple endocytic pathways in a given cell type exhibit different sensitivities to pro-oxidant inhibitors.

3. Inhibition of endocytosis by pro-oxidants also differs among different cell types.

4. Antioxidant phytochemicals can moderate the inhibition of endocytosis by pro-oxidants.

5. Decreased endocytic efficiency associated with aging differs in different cell types/tissues, and correlates inversely with the level of antioxidant capacity among those cells/tissues.

6. Physiological factors such as steroid hormones and growth factors can modulate the efficiency of endocytic transport, and such modulation differs among different endocytic pathways.

7. A pharmacological compound that likely prevents infection of cells by rickettsiae, can inhibit endocytic transport.

Chapter 2. MATERIALS AND METHODS

2.1. Biochemicals

Epidermal growth factor (EGF), human holo-transferrin (Tf), biotinylated Tf (bTf), streptavidin-peroxidase conjugate (streptavidin-HRP or ESa), peroxidase (HRP), o-phenylenediamine dihydrochloride (OPD), biotin N-hydroxy- succinimide (BNHS), human transthyretin (TTR), hemin, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. Protease inhibitors were purchase 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 ESI-09 compound, 3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-propionitrile, an EPAC inhibitor, was obtained from Dr. X. Cheng, Department of Pharmacology and Toxicology, University of Texas Medical Branch, UTMB. Vitamin D-binding protein (V/DBP) was obtained from Abcam.

Protein ligands such as DBP and TTR (not HRP) were biotinylated using BNHS (Biotin N-hydroxysuccinimide ester; Sigma) at a 5:1 reagent to protein molar ratio in PBS. After reaction for 30 min, TBS was added and the ligand stored frozen at -80^{o}C. Ligand-enzyme conjugates (ESa-bL) were prepared by incubating ESa with L at a ~ 1:1 molar ratio in PBS for 1 h at room temperature, typical incubation conditions previously reported (Shimada et al., 2005; Weitz-Schmidt et al., 1996; Vieira 1998; Graf and Friedl, 1999). Misfolded-aggregated TTR (agTTR) was prepared and characterized by standard procedures as reported previously (Fong and Vieira, 2013; Hammarstrom et al., 2002).

2.2. Cell and tissue preparations

Hepatocytes were prepared from mouse (BALB/c and CD-1) and fish livers using a chelator-based technique (Kravchenko et al., 2002). After perfusion of either the whole circulatory system or of the extracted liver with physiological saline, the gall bladder was removed, and the liver rinsed first with PBS then with EDTA-sucrose (ES) solution (250 mM Sucrose and 4 mM EDTA). In a plastic dish containing 5 ml of ES, the liver was chopped and torn into the smallest possible pieces, and placed for 5 min on ice. The tissue suspension was then passed five times through a 5-ml syringe (no needle), five times through a 1-ml pipette tip

with the tip (~ 8mm) cut off, and five times again through smaller tip opening (~4mm cut off). The suspension was collected into tubes, and dish further rinsed with 0.5-1.0 ml of ES, followed by a brief centrifugation of the tubes at low g-force (2 s, 50 x g) to pellet tissue debris. The supernatant was carefully transferred to new tubes which were subsequently centrifuged, 50 x g for 3 minutes to pellet the cells. (Additional liver cells could be obtained by re-suspending each tissue debris pellet in 1 ml of buffer and centrifuging for 2 s, 50 x g, followed by centrifugation of the supernatant for 50 x g for 3 minutes, as above).

Each liver cell pellet was resuspended in 1 ml of complete cell culture medium (DMEM-FBS) and re-centrifuged, 100 x g for 1 min to pellet cells. The cell pellet was then resuspended in about 2 volumes of PBS-BSA-SFM (PBS, pH 7.4, containing 1% w/v bovine serum albumin is mixed with equal volume of SFM), and kept at room temperature for about 10-30 min before being used for experiments. Cells were then re-centrifuged (100 x g, 1 min) and resuspended in the appropriate solutions for subsequent experiments.

For the preparation of semi-intact cell membranes (SICM), cells were rinsed 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). After centrifugation (14,000 x g, 30 s), the cell pellet was resuspended in an approximately equal volume of high sucrose-KSHM (100 mM potassium acetate, 0.75 M sucrose, 20 mM HEPES and 1 mM magnesium acetate) and frozen at -80°C (or on dry ice). The lysed cells were then pelleted, and the membrane

pellets rinsed in 1 ml of PBS. The pellets were again resuspended in high sucrose-KSHM and frozen as above. After another centrifugation, the membrane pellets were resuspended in 1ml of PBS. Then a final centrifugation was performed, and the membrane pellets were resuspended in an approximate, equal volume (to standardize protein concentrations) of the appropriate buffer to be used in subsequent experiments.

Tissues from mice (C57BL/6) were homogenized on ice in extraction buffer (EB, PBS with 0.1% Triton X-100, and protease inhibitors) at a ratio of 2 ml EB per gram of tissue. Tissue extracts were centrifuged at 12,000 x g for 5 min at 4°C. The resulting supernatants were collected and stored at -80^{o}C. In the current study, these extracts were used for oxidation assays (see below Section 2.3). Lung, liver, and kidney tissues were available for comparisons between young (1-2 mo.) and old mice (26-28 mo.). For middle aged mice, tissue extracts were prepared from lung, liver, kidney, adipose, and brain.

Human epithelial carcinoma cell line (A431, epidermoid cells) and Schwann cell-like line from human malignant peripheral nerve sheath tumor (sNF94.3) were obtained from American Type Culture Collection (ATCC). A431 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) and sNF94.3 cells in DMEM containing 1 mM sodium pyruvate. Both media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). Cells were maintained in humidified 5% CO2 incubator at 37^{o}C.

2.3. Assay methods

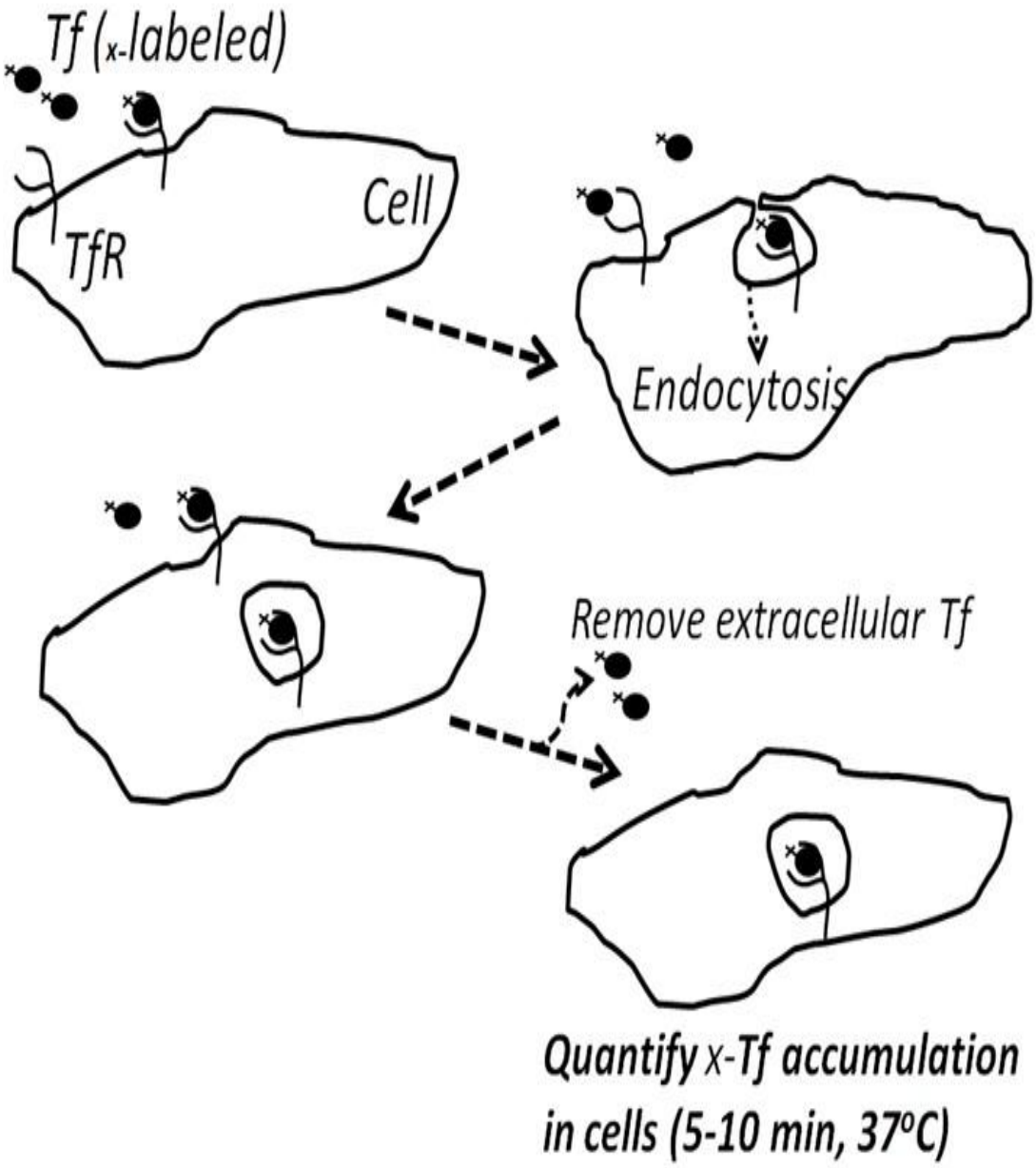
2.3.1. Endocytosis assays

Before addition of ligand and before any endocytic events in the mammalian cells, equal volumes of cell suspension were pre-treated for 30 min at 37^{o}C (unless acute treatment is mentioned) with the various pro-oxidant, anti-oxidant, or physiological factors in Eppendorf tubes. The concentrations of these factors are given in the Results section or Figure Legends. After this 30 min incubation, biotin-labeled ligand was added to the cell suspension on ice, and the tubes were incubated at 37^{o}C for 10 min to allow endocytosis. Cells were then returned to ice to stop endocytosis, and the tubes were centrifuged (14000 x g) for 15 s to pellet the cells. Tubes were placed back on ice, and the supernatants carefully removed. The cell pellets were then rinsed with 100µl of low-pH acetate buffer (pH 3) and re-centrifuged as above. The cell pellet was again washed with 100µl of low-pH buffer by re-suspending the pellet through the pipette tips 3 times. The tubes were then re-centrifuged as above, and the low pH acid was repeated. After removing the final acid wash, the pellets were re-suspended in 100ul of lysis buffer (1% Triton X-100, 0.1% SDS in 1 mM EDTA, 50 mM NaCl and 10 mM Tris-HCl, pH 7.4) and stored frozen until analysis of the biotin-labelled ligand as described below. Similar procedures were used for the trout liver cells at 15^{o}C (instead of 37^{o}C).

The internalized ligand was captured on anti-ligand IgG-coated plates and, after rinsing, tagged with streptavidin-peroxidase. The biotin-ligand was quantified using the standard ELISA colour reaction. This reaction involves 0.4 mg/ml o-phenylenediamine dihydrochloride (OPD) and 0.02% hydrogen peroxide in 50 mM Na2PO4 and 30 mM sodium citrate, pH 5. The colour reactions were terminated with 2 N sulfuric acid. Absorbance was measured at 490 nm (ELISA plate reader). Lysates from endocytosis assays performed with HRP ligand were analyzed directly by the OPD colour reaction.

Figure 2.1. In vitro transferrin (Tf) endocytosis assay, a clathrin-dependent transport activity.

After treatment of cells with potential endocytic modulators, biotin-labeled transferrin (x-Tf) was added to cells and internalized (10 min, 37^{o}C, in the current experiments). After removal of non-internalized x-Tf, the cells were lysed, and the extracts subjected to quantification of x-Tf. The method was similar for SICM (semi-intact cell membrane) ligand-sequestration assays, but these included cytosolic factors and an energy source (ATP). Although the schematic shows internalization of transferrin, the same type of assay was used for other biotin-labelled ligands such as VDBP.



2.3.2. Binding assays

Cells were prepared as described above for the endocytosis assays (Section 2.3.1), but the ligand was added to the cells on ice and the binding incubation was performed at a temperature of 4°C (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 by pellet resuspension, 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 for total binding on anti-Ligand IgG-coated plates as described above for endocytosis.

2.3.3. Oxidation assays

The assays were performed on 96-well plates. The final concentration of reagents in the assay was as follows: 1 µM hemin, 1 mM H_2O_2 and 100 µM TMPD (N,N,N',N'-tetramethyl-1,4-phenylenediamine). Tissue (or cell) lysates were added into the wells, 10 µl/well, containing hemin. Buffer used to prepare the lysates was used as a control. Subsequently, hydrogen peroxide, and then TMPD was added to the wells. Total well volume was 50 µl. Absorbance

measurements were made at 590 nm using an ELISA plate reader at 1, 2, 4, and 8 minute reaction times.

2.4. Statistics

Results were 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 its control. The statistical package JMP10 was used. Tukey's Multiple Comparisons method was used where applicable. Confidence intervals were standard at 95% confidence intervals unless otherwise stated.

Chapter 3. RESULTS

3.1. Effects of pro-oxidants and aging on endocytosis

3.1.1. Effects of pro-oxidants on transferrin CME in different cell types

I have performed experiments to examine how selected pro-oxidants affect endocytic pathways. **Figure 3.1.1** provides a comparison of two pro-oxidants, iron-ascorbate and hydrogen peroxide, on the CME pathway of Tf. These pro-oxidants were tested at concentrations that have been used in other, reported studies. At the indicated concentrations both treatments result in a significant inhibition of about 20% relative to untreated controls ($p<0.05$).

The effects of this pro-oxidant treatment (0.5 mM hydrogen peroxide, Px), on the same transferrin endocytosis pathway, were compared in three different cell types: fish hepatocytes, mouse hepatocytes, and a human epidermoid cell line (A431). As shown in **Figure 3.1.2**, this pro-oxidant was inhibitory in all cell

types, and most inhibitory in the human carcinoma line. There was a statistically significant difference ($p < 0.05$) between the effects of Px on primary murine hepatocytes and the A431 epidermoid cells, and also between intact A431 cells and semi-intact A431 (membrane preparations, see Methods). Another comparison using values reported in the literature (Cheng and Vieira, 2006; Ihara et al., 2002), yields similar values for inhibition of transferrin endocytosis by 0.1 mM hydrogen peroxide: 34-37% inhibition relative to controls without the peroxide. **Table 3.1** shows my results and provides a comparison with previous studies of peroxide-induced inhibition of endocytosis.

Figure 3.1.1 Inhibition of transferrin endocytosis by iron-ascorbate and hydrogen peroxide in murine hepatocytes.

Concentrations used were similar to those in other studies, 10 μM Fe + 100 μM Asc, and 0.5 mM Px; n=4-6 experiments for each condition. $P < 0.05$ for each pro-oxidant treatment relative to untreated (buffer) controls.

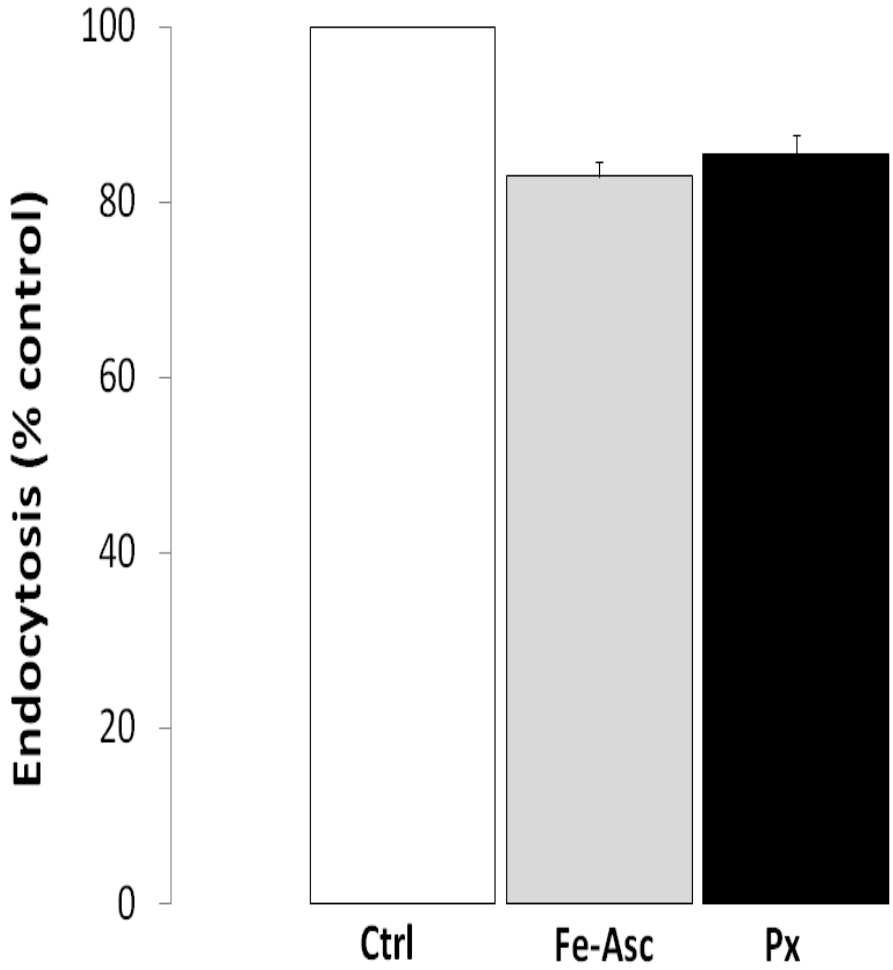


Figure 3.1.2 Comparison of hydrogen peroxide effects on the CME of transferrin in three different cell types.

Piscine and murine hepatocytes, and a human epidermoid cell line (A431) were all inhibited by H_2O_2 (0.5mM). In addition, there was statistically significant difference between the mouse and A431 cells ($p < 0.05$). *n* = 4-9 experiments for each condition.

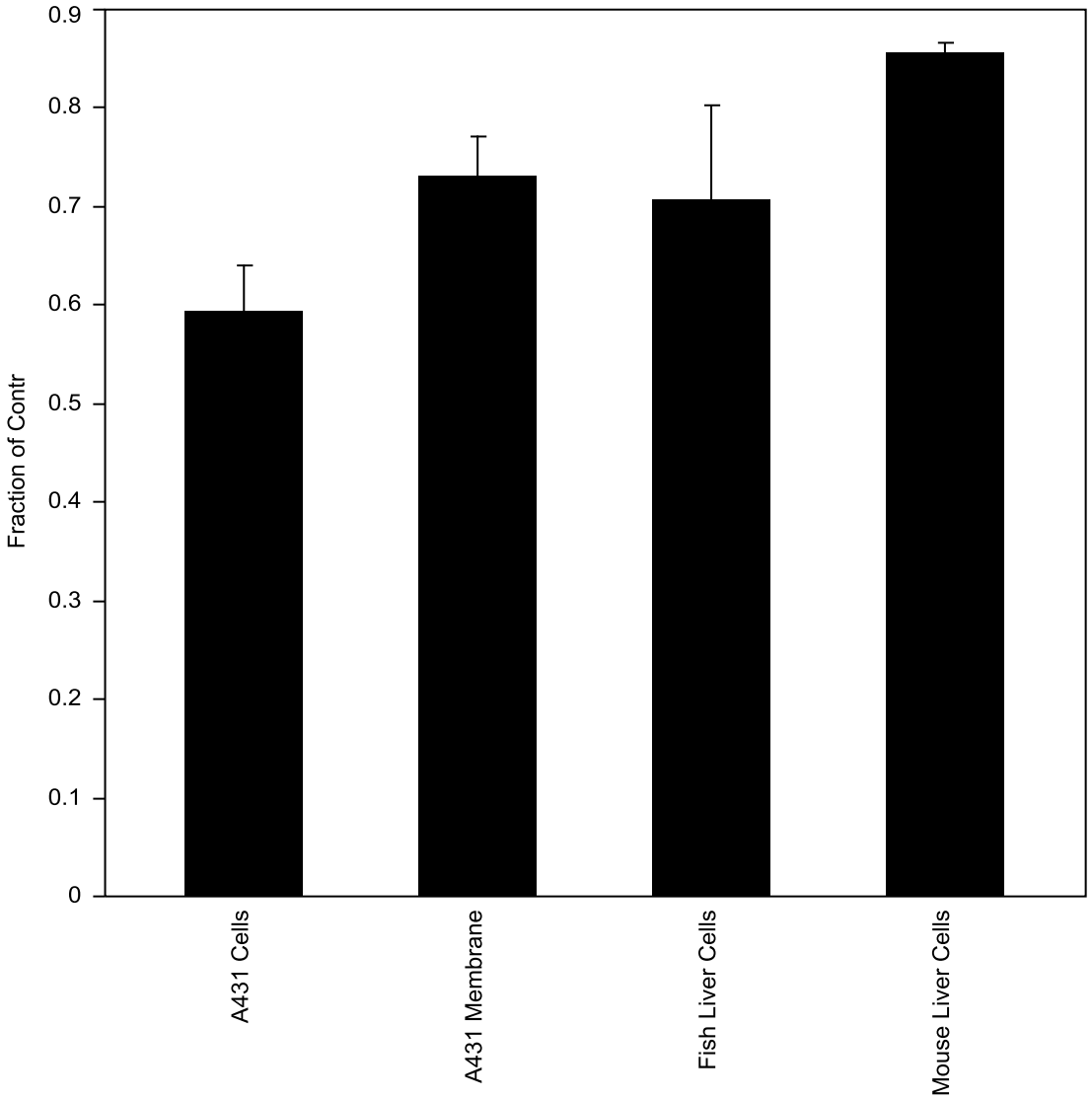


Table 3.1. Comparison of inhibitory effects of hydrogen peroxide (Px) on endocytosis of different ligand/receptor systems.

References: 1, Ihara et al., 2002; 2, Cheng and Vieira, 2006; 3, Current study; 4, de Wit et al., 2001; 5, Kano et al., 2011. The indicated studies analyzed the internalization of ligand (Tf, EGF) or of the receptor (EGFR) as indicated.

[Px]	**Ligand/ Receptor**	**% control** (reported ranges)	**Ref.**
0.1 mM	Tf	63-66	1, 2
0.5 mM	Tf	56-70	1-3
	EGF/EGFR	40-72	4, 5
2.5 mM	Tf	23-50	2
	EGF	15-20	4, 5

3.1.2. Effects of pro-oxidants on different endocytic pathways

To assess how the same pro-oxidant may affect different endocytic pathways in the same cell type, the internalization of Tf and HRP was compared. **Figure 3.2.1** shows the relative effects of hydrogen peroxide (Px) and iron-ascorbate (Fe-Asc) on CME (Tf ligand) and on non-specific endocytosis (HRP ligand) in mouse liver cells. While Px inhibits HRP more than Tf; the opposite is true for Fe-Asc ($p < 0.05$). In comparison, fish liver cells (**Figure 3.2.2**) did not show significant effects ($p > 0.05$) of Px or of a peroxide-heme combination (PxHm) on Tf/HRP ratio. However, in terms of non-specific HRP uptake pathways tested in primary mouse and fish liver cells, Px was also inhibitory. As shown in **Figure 3.2.3**, HRP uptake inhibition by 0.5 mM Px was 60-65 % of control for both the murine and piscine liver cells ($p < 0.05$). The difference between the Px inhibition of these two liver cell types was not statistically significant ($p > 0.05$). By comparing **Figure 3.1.2** and **Figure 3.2.3**, it can be concluded that mouse liver cells exhibited a greater inhibition by Px on HRP uptake relative to Tf uptake (20 % more inhibition for HRP, $p < 0.05$).

Figure 3.2.1 Relative effects of hydrogen peroxide and iron-ascorbate on the transferrin and HRP endocytic pathways in mouse liver cells.

The former pathway is CME; the latter represents non-specific endocytic pathways. Px, 0.5 mM; 10 μM Fe + 100 uM Asc; *n* = 5 experiments for each condition. *P* < 0.05 for each pro-oxidant treatment ratio relative to control ratio.

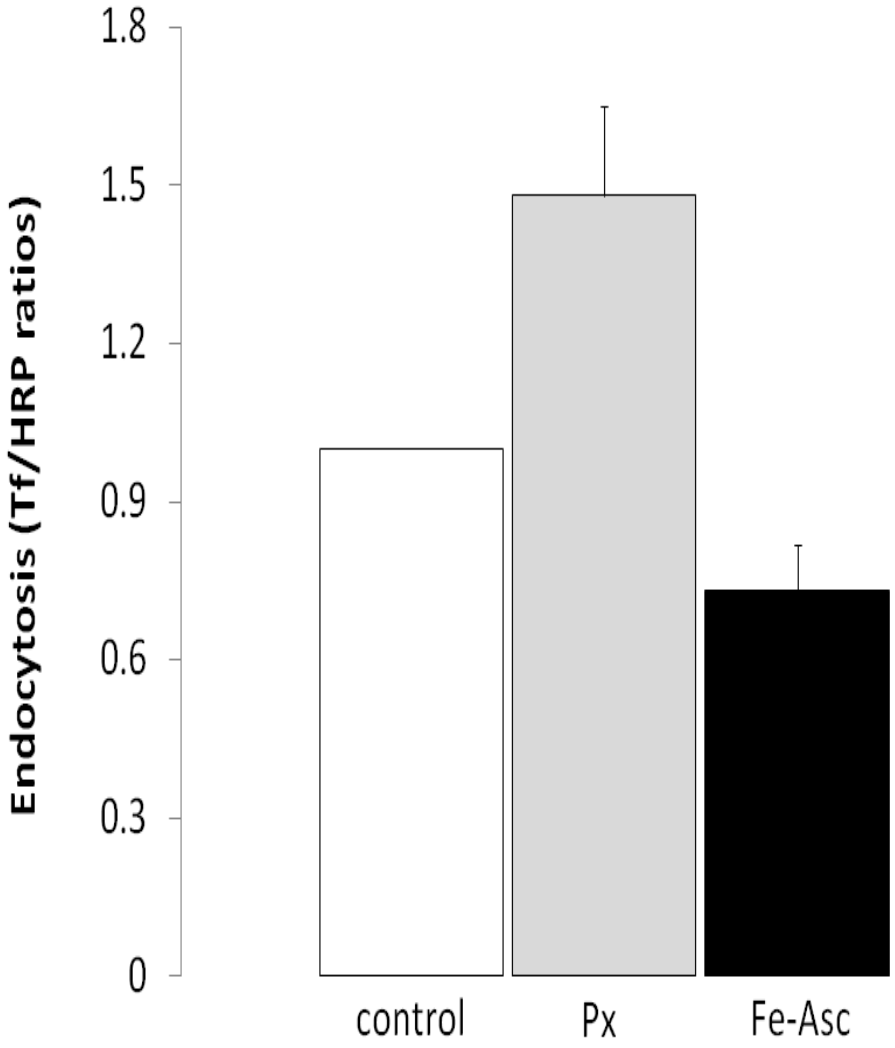


Figure 3.2.2 Relative effects of peroxide (Px) and peroxide + hemin (PxHm) on the transferrin and HRP endocytic pathways in fish liver cells.

The former pathway is CME; the latter represents non-specific endocytic pathways. Px, 0.5 mM; PxHm, 0.5 mM Px + Hemin; *n* = 4 experiments for each condition. *P* > 0.05 for each pro-oxidant treatment ratio relative to control ratio.

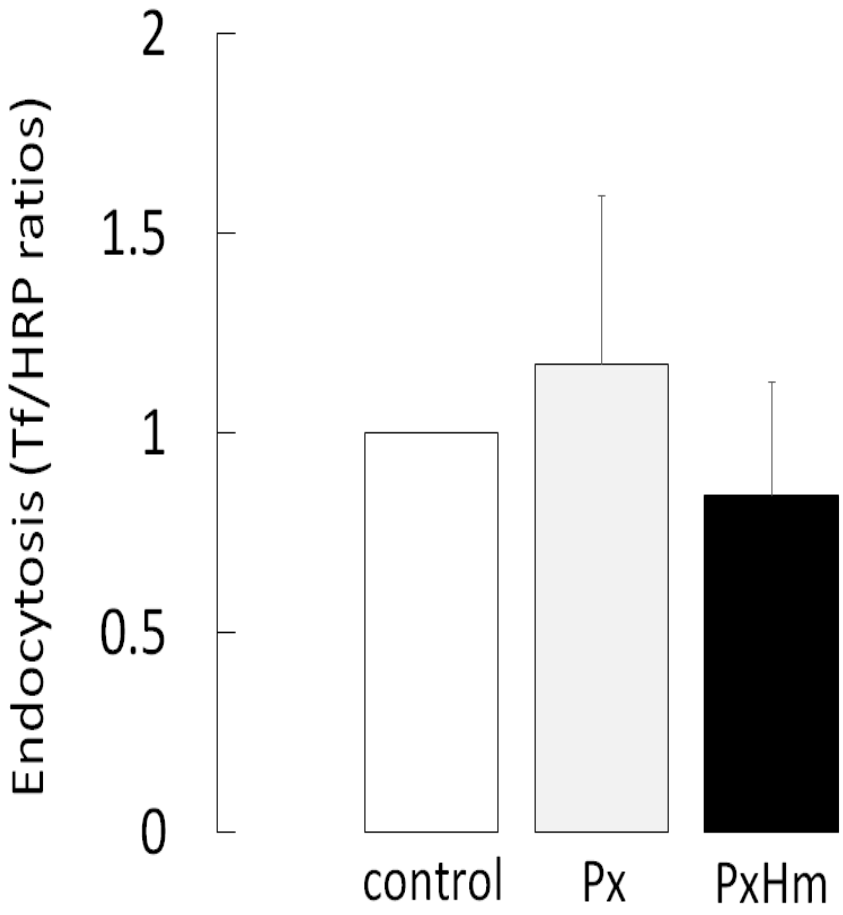
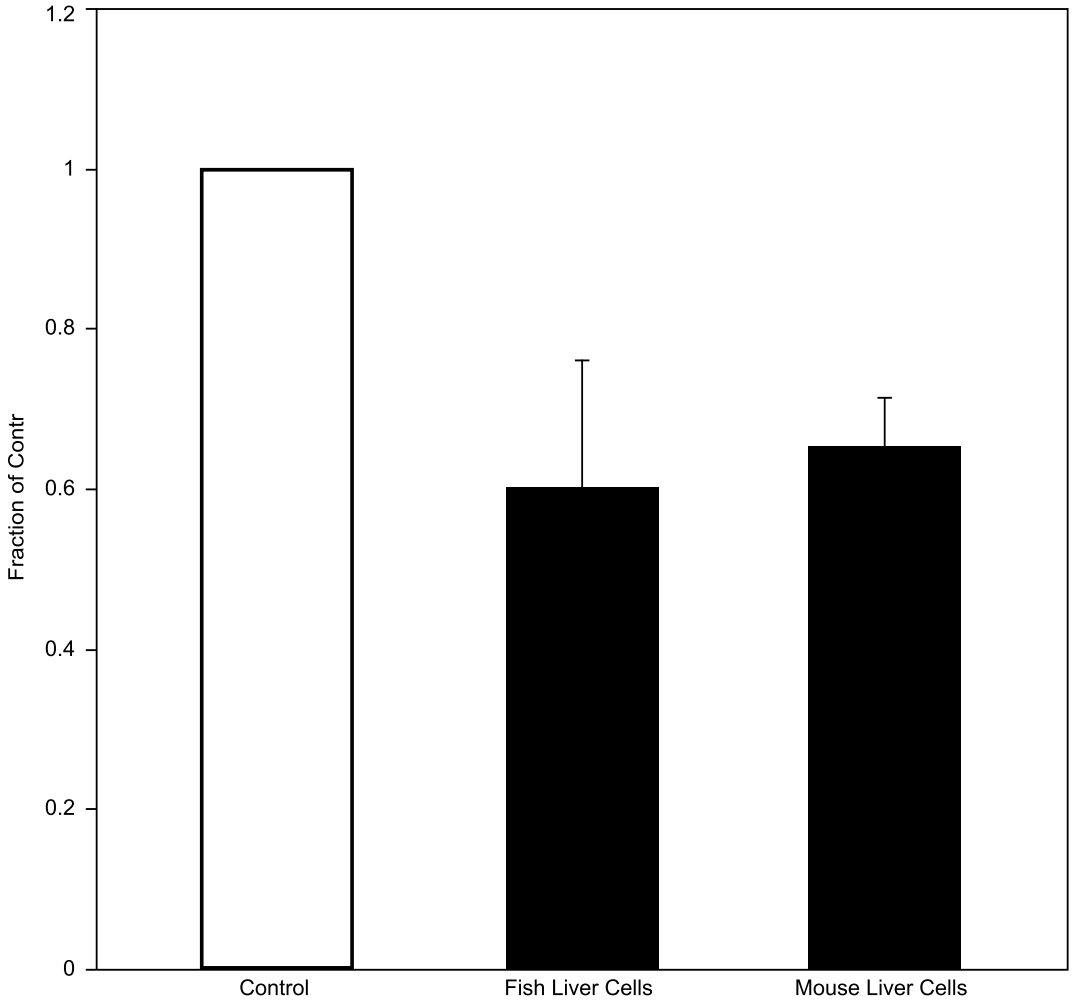


Figure 3.2.3 Comparison of hydrogen peroxide (Px) effects on the endocytosis of HRP in different cell types

Fish hepatocytes and mouse hepatocytes were both significantly inhibited by Px (0.5 mM, $p < 0.05$ relative to buffer control). There was no statistically significant difference between the two cell types ($p > 0.05$); *n* = 4-7 experiments for each condition.



3.1.3. How pro-oxidative protein aggregates associated with aging affect endocytosis

Misfolded, aggregated transthyretin (agTTR) is a pathological factor involved in age-related amyloidogenic disease, and has pro-oxidative effects on cells including increased production of hydrogen peroxide (Ando et al., 1997; Fong and Vieira, 2012; Sousa et al., 2001).

Figure 3.3.1 shows that, unlike the normal, soluble TTR, agTTR can result in a general (non-specific HRP uptake pathway) inhibition of endocytosis in the hepatocyte experimental model, 30% inhibition relative to normal TTR ($p < 0.5$ in paired t-test). A comparison was made of the inhibitory effects of agTTR on murine hepatocytes and on a human epidermoid cell line. **Figure 3.3.2** shows that agTTR can inhibit endocytosis in both cell types to approximately the same extent.

Figure 3.3.1 Inhibition of HRP endocytosis by agTTR, but not by normal, soluble TTR.

Control represent 100% endocytosis value and was obtained without any TTR addition (buffer only). TTR and agTTR were tested at 2 μM concentrations (pre-aggregation concentrations where applicable); *n* = 6 experiments for each condition. P < 0.05 for agTTR relative to the normal TTR.

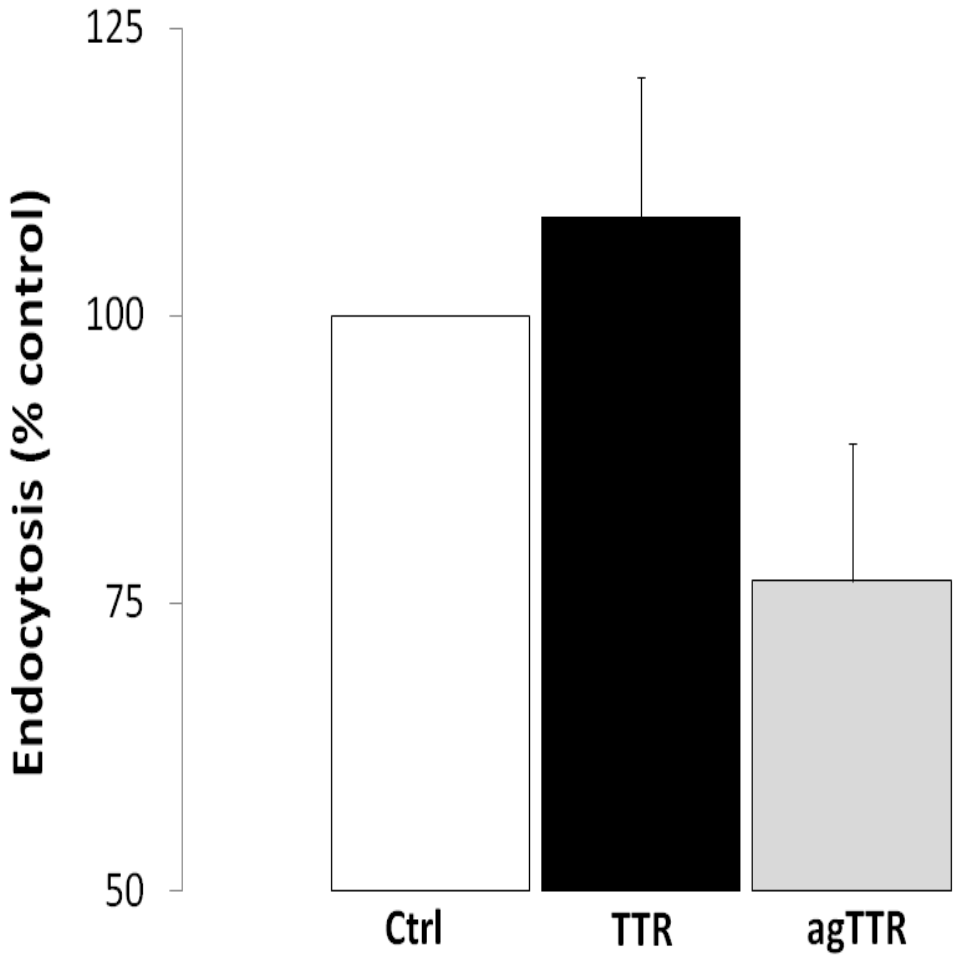
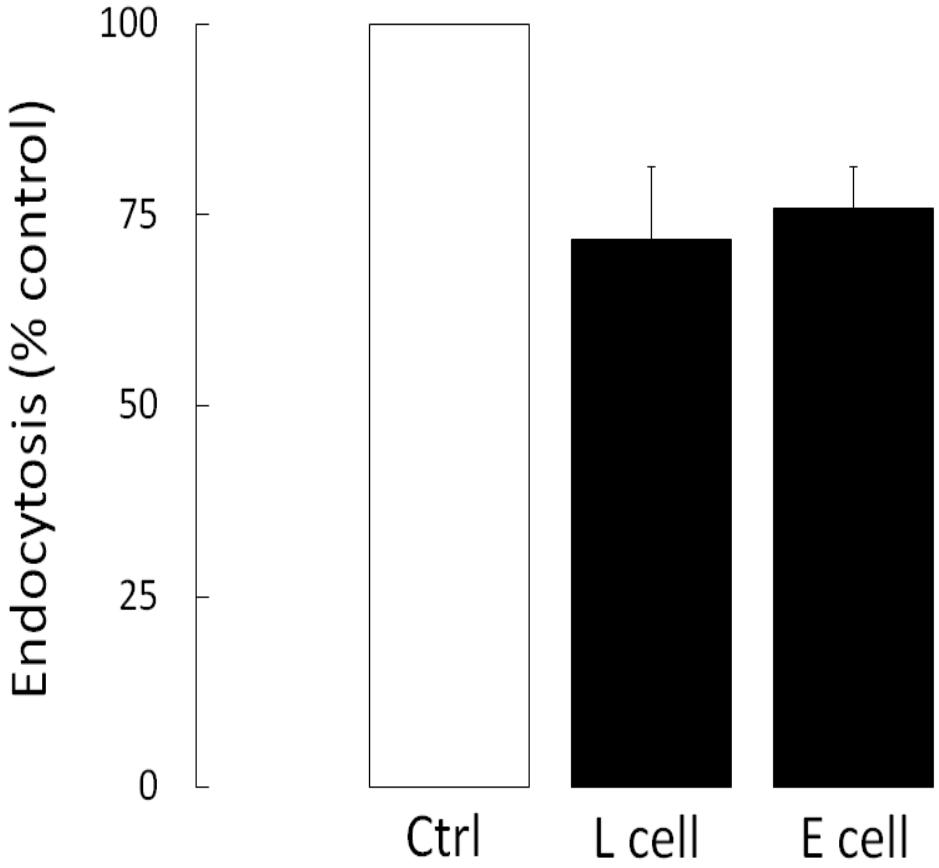


Figure 3.3.2 Inhibition of endocytosis by agTTR in two different cell types:

murine primary liver (L) cells and human epidermoid (E) carcinoma A431 cells. The results for both cell types relative to control (normal soluble TTR treatment) are significant ($p < 0.05$). TTR and agTTR were tested at 2 μM concentrations (pre-aggregation concentrations where applicable); *n* = 6 for each condition.



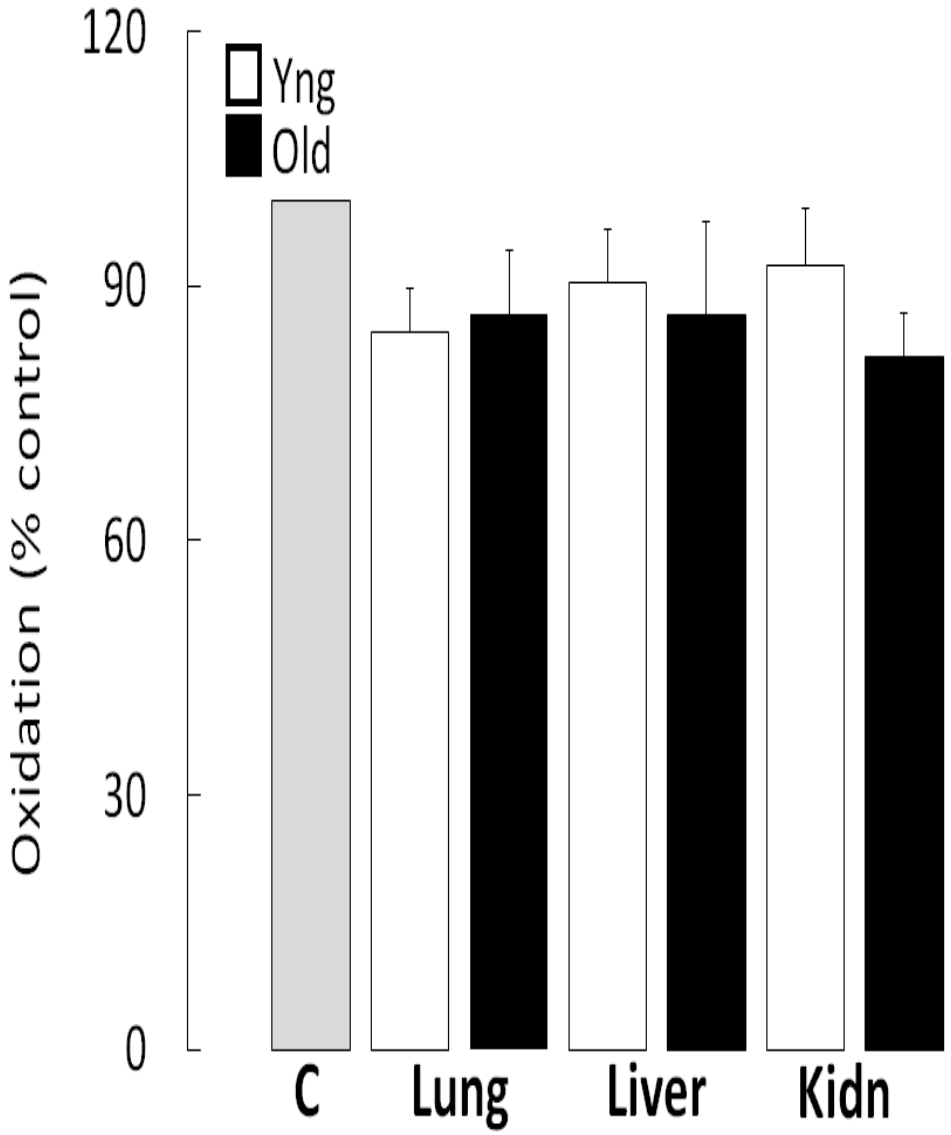
3.1.4. How is endocytosis affected by aging?

Table 4.1 provides examples of possible aging effects on endocytosis in different experimental systems. To test the possibility that oxidative stress may contribute to the age-related decrease in such transport, I tested the antioxidant capacity of mouse tissue extracts from young (1-2 months) and old (approximately 26-28 months) animals. These values from the oxidation assays were then compared to those from the transport assays for the same tissue extracts. A standard assay was used based on the oxidation of N N N'N'-tetramethyl-1,4-phenylene-diamine, TMPD (Yao and Vieira, 2007).

Figure 3.4.1 shows a lower extent of oxidation in all the samples that contained tissue extracts. But contrary to was expected, the extracts from old tissues did not contribute to higher levels of oxidation in this assay. The differences between each respective young and old tissue was not statistically significant ($p > 0.05$).

Figure 3.4.1. Comparison of oxidation in assays containing tissue extracts from old and young animals.

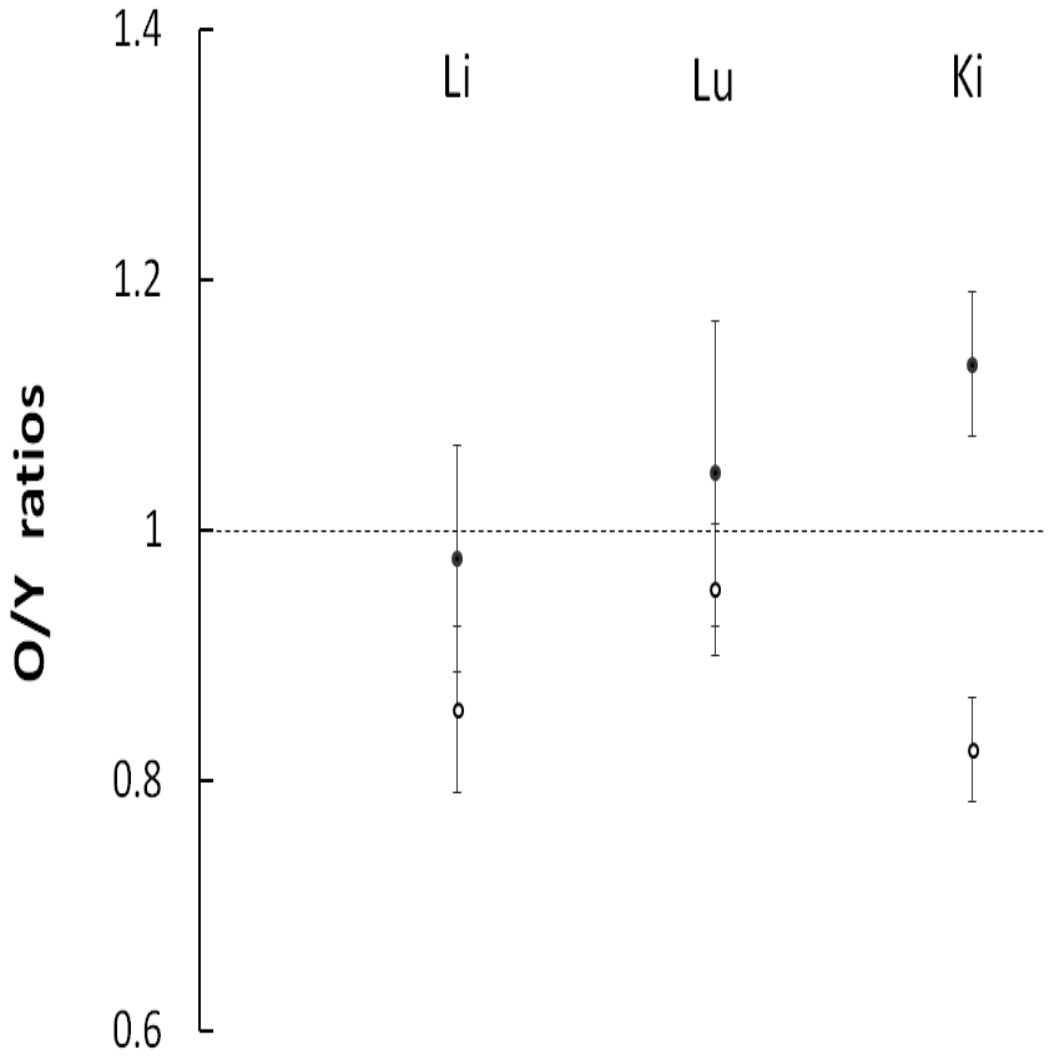
Control represents assays with all reagents except tissue extracts; n = 6 experiments for each condition. $p > 0.05$ between young and old tissues. As described in Materials and Methods this assay was based on the oxidation of TMPD and included both hydrogen peroxide and hemin.



The inverse of the oxidation values (represents antioxidant capacity, AOC) for each tissue was then plotted in on a graph along with estimated endocytic efficiency for the same tissue extracts. Old-to-young (O/Y) ratios were plotted to facilitate comparisons of the data. O/Y ratios were expected to be < 1 for both sets of data. **Figure 3.4.2** shows the expected lower transport values for the old tissues (all ratios below 1); but the AOC ratios for two of the old tissues (lung and kidney) are over 1 and the third (liver) is just below, but very close to 1. Kidney tissue extracts exhibit the largest age-dependent inhibition in transport and, unexpectedly, the same kidney extracts also exhibit the largest age-dependent increase in AOC.

Figure 3.4.2. Comparisons of estimated endocytic efficiency and antioxidant capacity (AOC).

The data is presented as Old/Young (O/Y) ratios for three tissues, liver (Li), lung (Lu), kidney (Ki). Estimated endocytic efficiency (open circles, three lower points) was determined by quantifying a biotin-labelled internalized ligand (bRBP, from Table 3.2) 20-30 min post-injection. AOC (inverse of oxidation, solid circles) was determined in oxidation assays (see Materials and Methods), and calculated using the data for Figure 3.4.1; AOC ratios for Lu and Ki are > 1; n = 5.

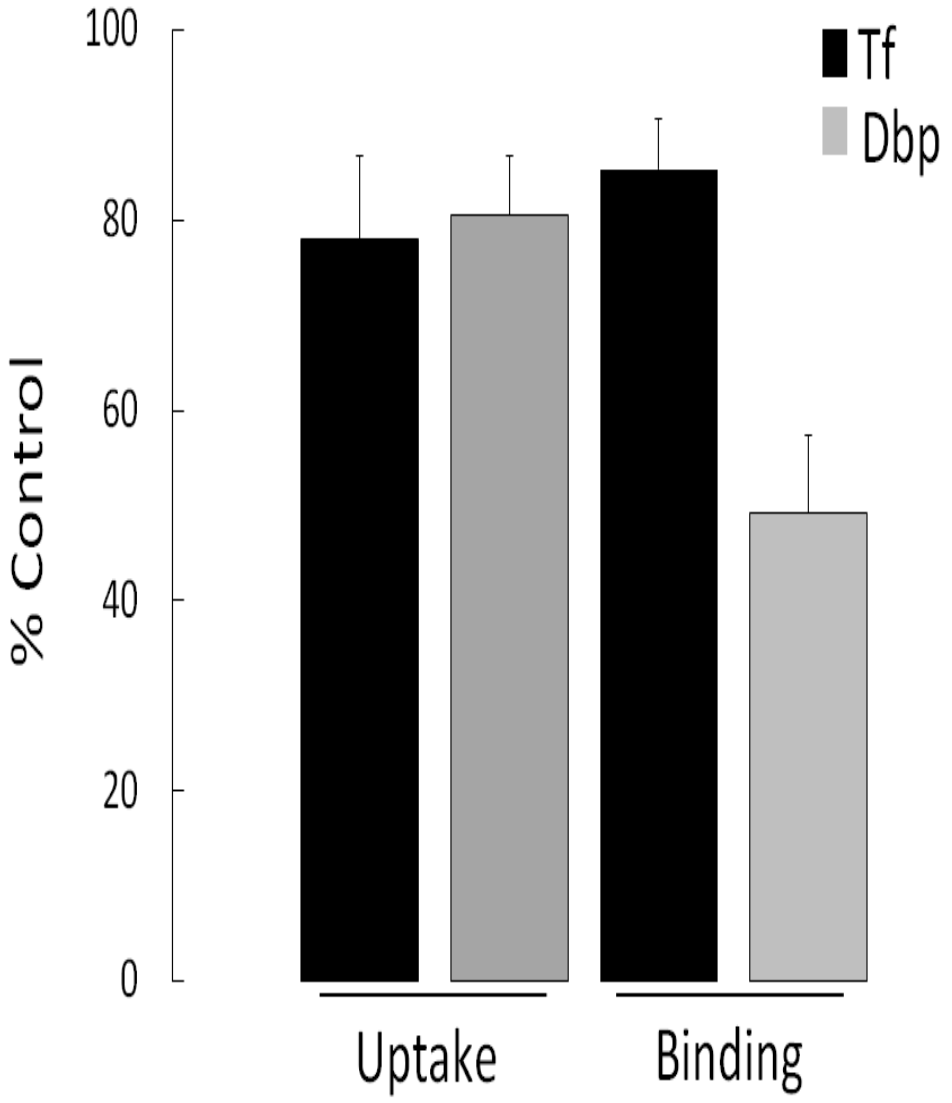


3.1.5. How is endocytosis affected by redox-active vanadate ions?

As a link between redox and cell signalling effects (details in Discussion), vanadate ions were tested for possible modulation of endocytic pathways including that of vitamin D binding protein (VDBP) and the CME of Tf. **Figure 3.5.1** shows an inhibition of total VDBP binding to hepatocytes treated with vanadate ($p < 0.05$ relative to Tf). But the uptake of the ligand over the 10 min time period of the assay was not significantly affected by vanadate ($p > 0.05$).

Figure 3.5.1. Effects of vanadate pre-treatment (30 min) on the cell surface binding and endocytosis of Tf and VDBP in mouse liver cells

n = 5 for each experimental condition; 2 microM vanadate. VDBP (DBP) total binding was significantly inhibited by vanadate relative to Tf binding (or non-vanadate-treated controls; $p < 0.05$).



3.2. Modulation of endocytosis by hormonal and growth factors

3.2.1. Effects of EGF on hepatocyte endocytosis

I examined how cell treatments with EGF can affect the uptake of other CME ligands such as transferrin, and of non-CME ligands such as VDBP. **Figure 3.6.1** shows that pre-treatment of cells (30 min), but not an acute treatment (10 min exposure concomitant with endocytosis), with EGF can result in lower uptake of Tf. The inhibition that resulted from the 30 min treatment was small, about 15% below controls without EGF, but was significantly different from acute treatment ($p < 0.05$).

Possible effects of EGF treatment (30 min) on uptake of other ligands was also tested in the mouse liver cells. As shown in **Figure 3.6.2**, no statistically significant effect of EGF on the uptake of DBP or HRP was observed in these cells; the only significant difference relative to controls was for Tf endocytosis ($p < 0.05$).

Figure 3.6.1 Effects of EGF pre-treatment for 30 min (black bar) or acute presence of EGF (grey bar) on the 10 min, 37^{o}C endocytosis of transferrin in mouse liver cells.

EGF concentration in both cases was the same, 10 nM; $p < 0.05$ in comparing the two EGF treatments; n = 5-6 for each experimental condition.

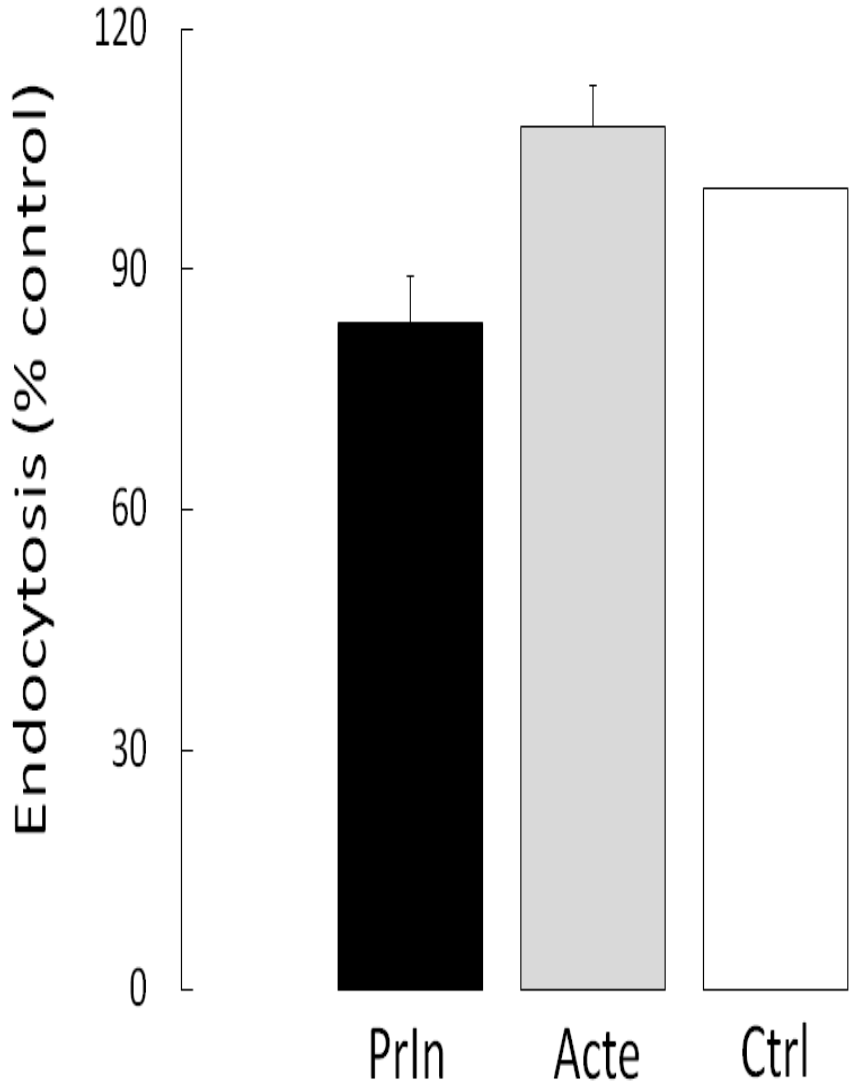
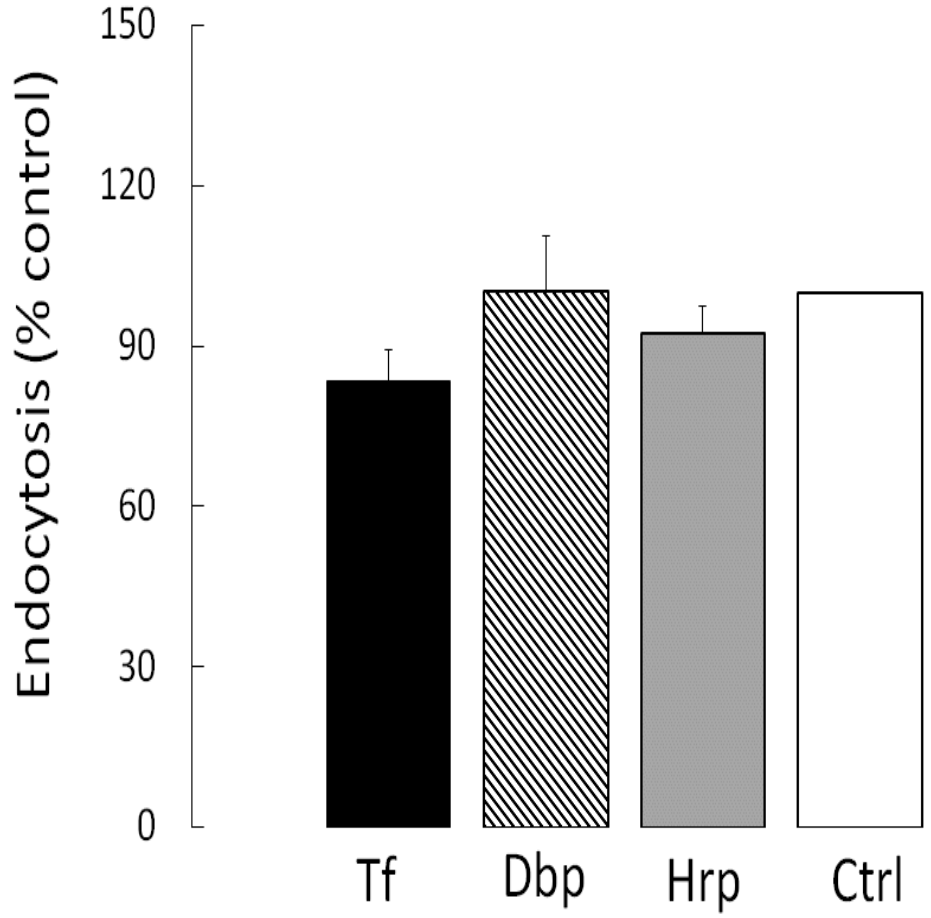


Figure 3.6.2. Effects of EGF pre-treatment (30 min) on the endocytosis of Tf, VDBP, and HRP in mouse liver cells

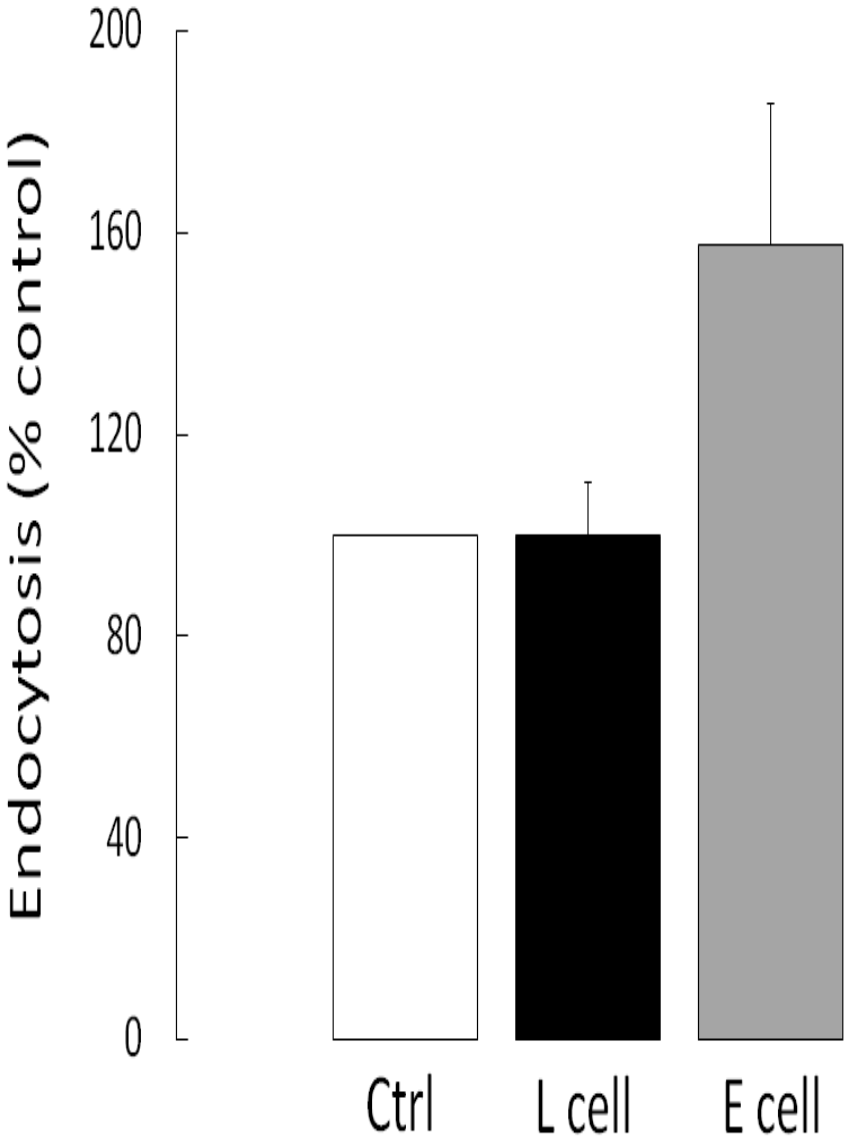
n= 6-8. As reported in the main text, only Tf endocytosis was significantly ($p < 0.05$) affected by EGF treatment relative to non-EGF controls (buffer only, 100% Ctrl). The two other ligands were not significantly affected by EGF treatment relative to control ($p > 0.05$)



A comparison was made of the effects of EGF on VDBP endocytosis in two different cell types: primary mouse liver cells and the A431 human epidermoid carcinoma line. As shown in **Figure 3.6.3**, there is a significantly greater EGF-mediated stimulation of VDBP uptake in the epidermoid cells (E), about 50% greater than in untreated controls or treated liver (L) cells ($p < 0.05$).

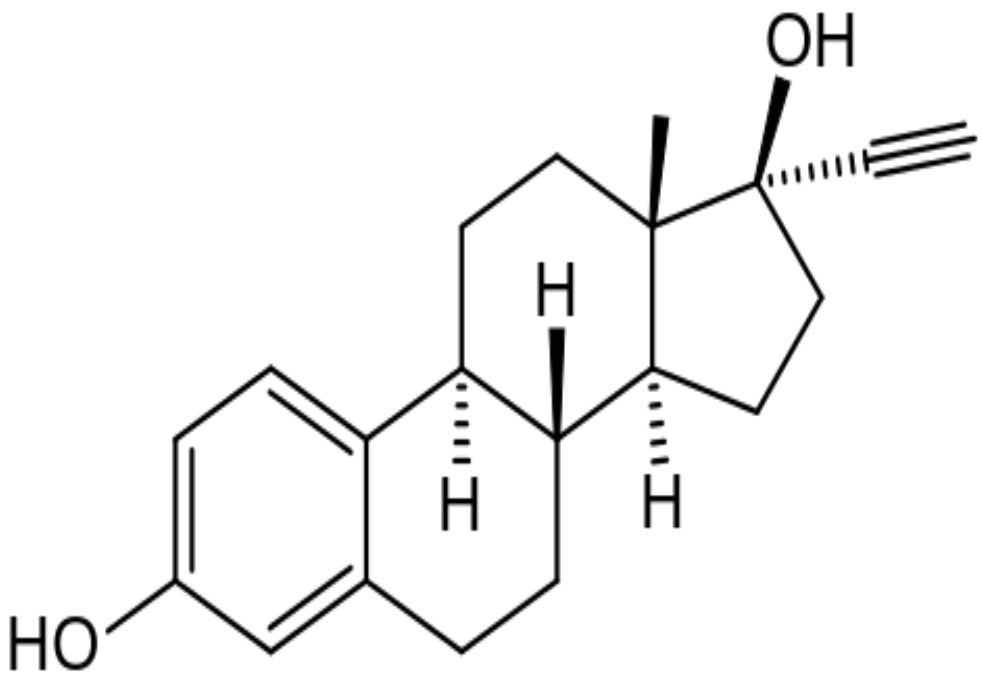
Figure 3.6.3. Comparison of the EGF stimulation of VDBP endocytosis in two cell types:

primary murine liver cells (L) and human epidermoid (E) A431 cells. The latter is significantly different relative to non-EGF-treated controls (buffer only) and EGF-treated liver cells ($p < 0.05$); $n = 6$ for each experimental condition.



3.2.2. Effects of the synthetic estrogen 17-alpha-ethinyl estradiol on endocytosis

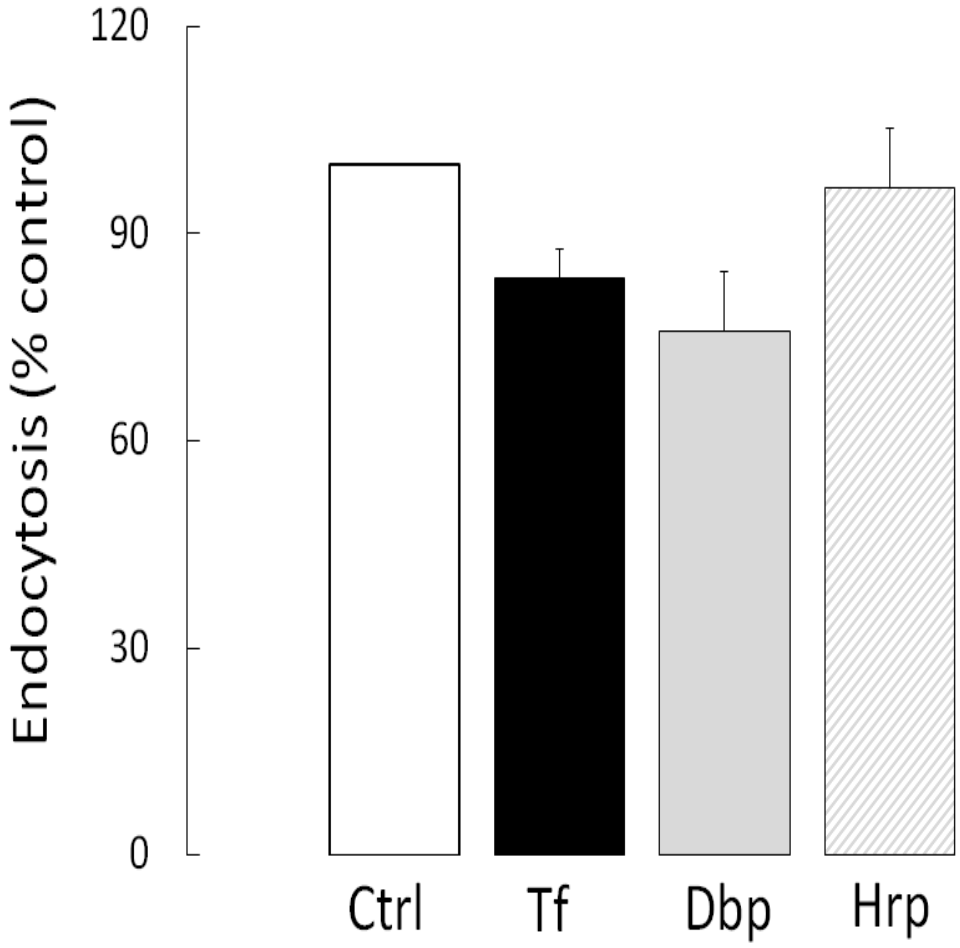
Figure 3.7.1. Chemical structure of 17- alpha-ethinyl estradiol.



The possible modulation of transport by the synthetic estrogen, 17- alpha-ethinyl estradiol (EE, 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol), was also examined. Different endocytic pathways—CME (transferrin), a specific non-CME pathway (VDBP), and non-specific endocytosis (HRP)—were tested. **Figure 3.7.1** shows that relative to control, endocytosis was significantly ($p < 0.05$) inhibited in the CME pathway as shown by decreased transferrin uptake (16% decrease) and in a specific non-CME pathway as shown by decreased VDBP uptake (24% decrease). There was a statistically insignificant difference for the inhibition of non-specific endocytosis (HRP) relative to untreated controls, and relative to Tf and VDBP, $p > 0.05$.

Figure 3.7.2. Effects of 17-alpha-ethinyl estradiol (EE) on different endocytic pathways.

Endocytosis of transferrin (Tf, specific CME pathway), vitamin D-binding protein (DBP, specific non-CME pathway) and peroxidase (HRP, non-specific pathways) were analyzed; n=5-7 for each experimental condition. Tf and DBP uptake was significantly inhibited by EE treatment relative to non-EE-treated controls, $p < 0.05$.



3.3. Modulation of endocytosis by phytochemical and pharmacological factors

3.3.1. Effects of apocynin on hepatocyte endocytosis

The structure of apocynin (acetovanillone) is shown below in **Figure 3.8.1**. There is reported antioxidant activity for this phytochemical compound in some assays (**Table 3.2** and references therein). To confirm such activity in a different assay (not previously reported for apocynin), I tested it in a hemin-peroxide oxidation assay (see Materials and Methods). As shown in **Figure 3.8.2**, apocynin did not exhibit statistically significant antioxidant activity at the concentrations tested (2-20 μM). This result and those from other, reported assays are summarized in **Table 3.2** for comparison.

Figure 3.8.1. Apocynin

(1-(4-Hydroxy-3-methoxy-phenyl)ethanone) is a phytochemical that has been isolated from various plant sources, originally from a Himalayan plant, *Picorhhiza kuroa*.

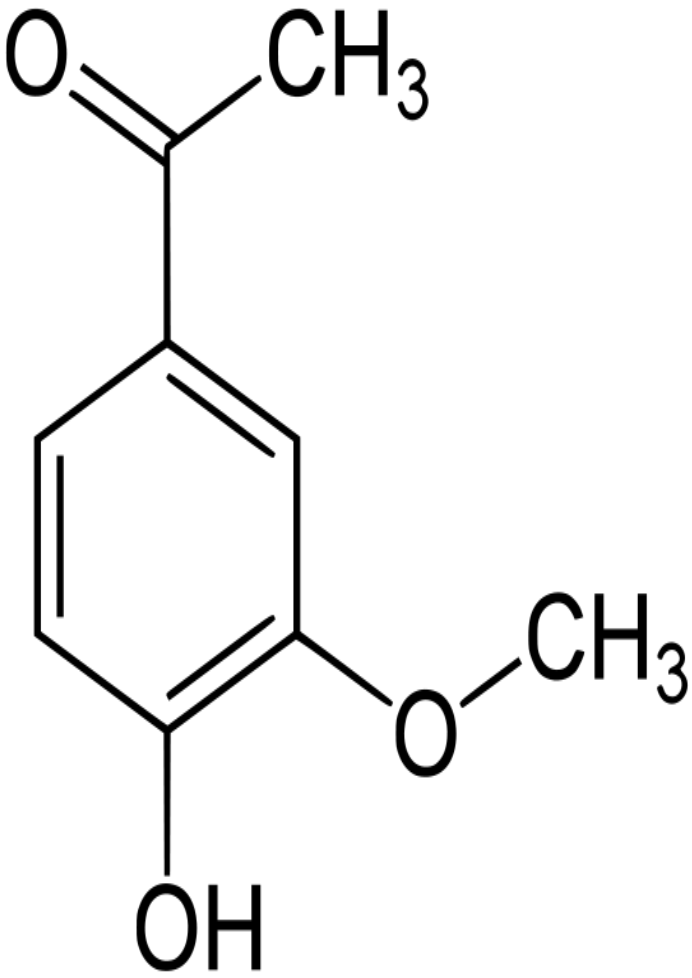


Figure 3.8.2. Antioxidant activity of apocynin in peroxide-heme oxidation assays.

Concentrations of apocynin, 2 and 20 μM, were tested (log scale shown). No statistically significant differences relative to control (no apocynin, buffer only) were observed ($p > 0.05$).

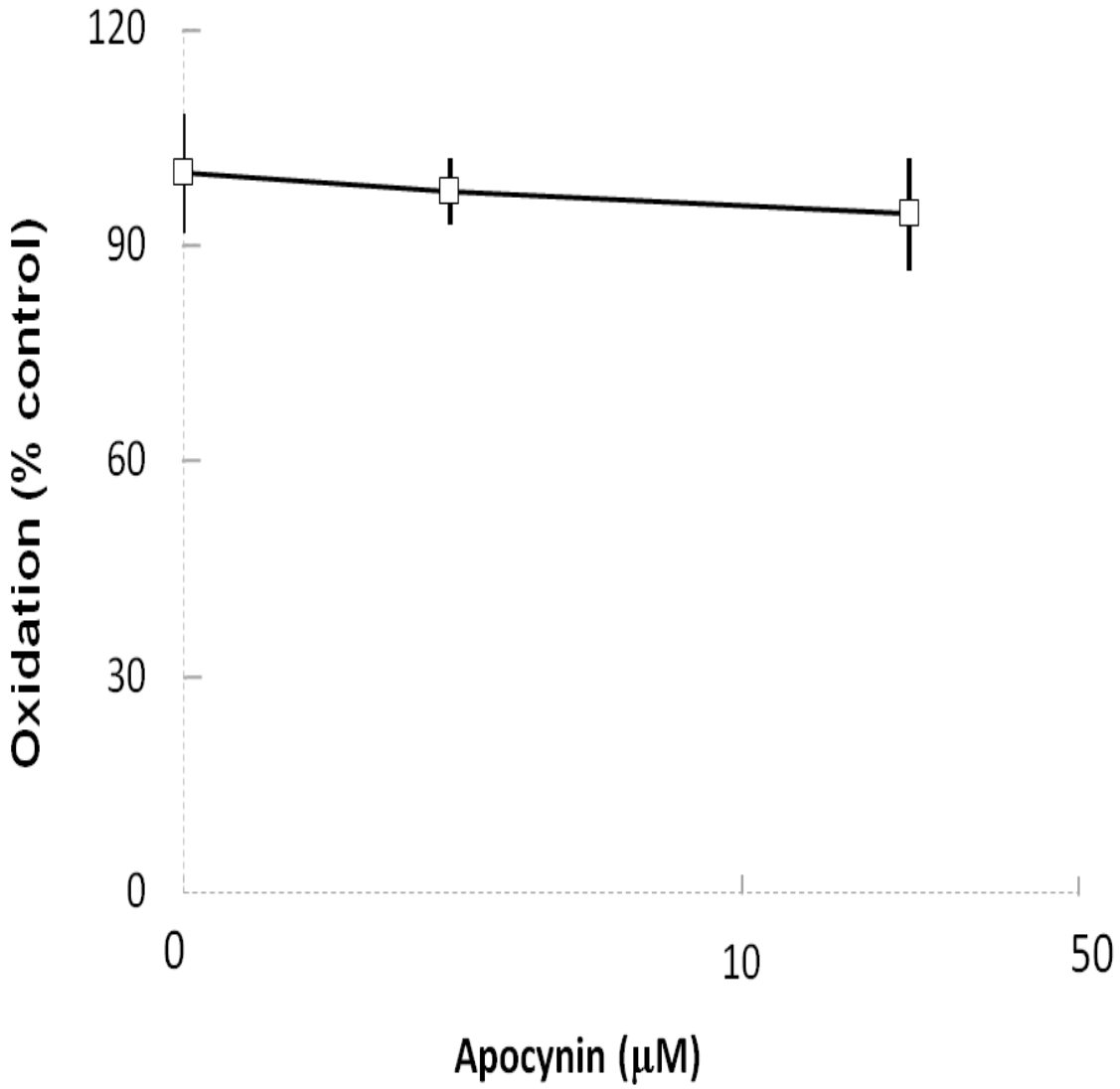


Table 3.2. Comparison of apocynin effects on oxidative stress or ROS production.

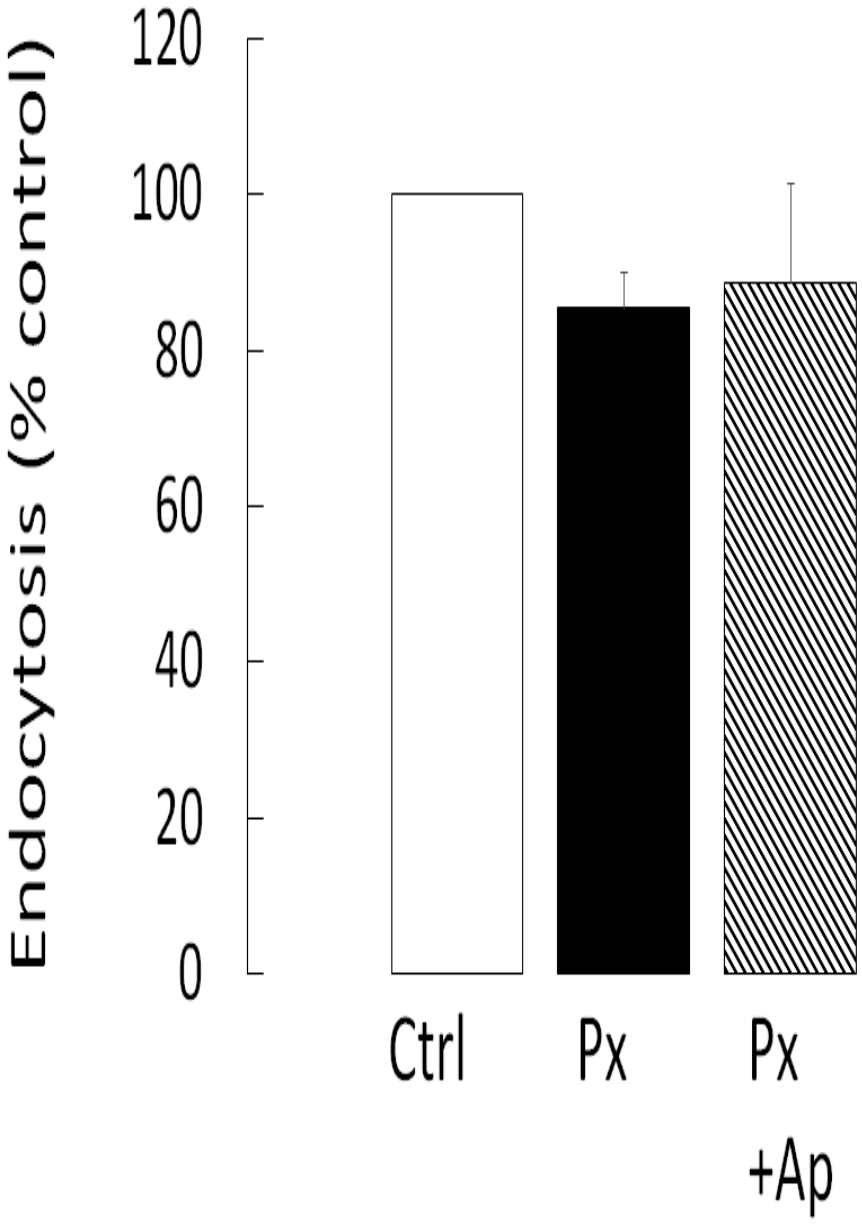
References are as follows: 1, Current study; 2, Lomnitski et al., 2000; 3, Khanicheh, et al., 2013 ; 4, Heumuller et al., 2008; 5, Fong (2012). Standard deviations were less than 12% for these studies.

Assay [apocynin concentration, if applicable]	**% control**	**Ref.**
Oxidation: Heme-hydrogen peroxide (Hm-Px), [20 μM]	~100	1
Oxidation: LPS: oxidative damage to murine tissues	~70	2
Oxidation: Hydroethidine; aortic superoxide generation	~ 100	3
Oxidation: PMA: leukocyte superoxide production [10 μM]	~75	4
Membrane structure: Filipin binding to A431 membranes (Hm-Px)	~110	5

When tested against the inhibition of endocytosis by hydrogen peroxide, apocynin did not exhibit rescue activity. As shown in **Figure 3.8.3**, in the presence of peroxide, apocynin at 20 μM did not show a statistically significant effect upon transferrin endocytosis compared to peroxide alone.

Figure 3.8.3. Effects of apocynin on the hydrogen peroxide-mediated inhibition of Tf endocytosis in mouse liver cells.

The presence of apocynin (20 microM, Ap) did not significantly alter endocytosis relative to assays performed with peroxide alone (0.5 mM, Px).



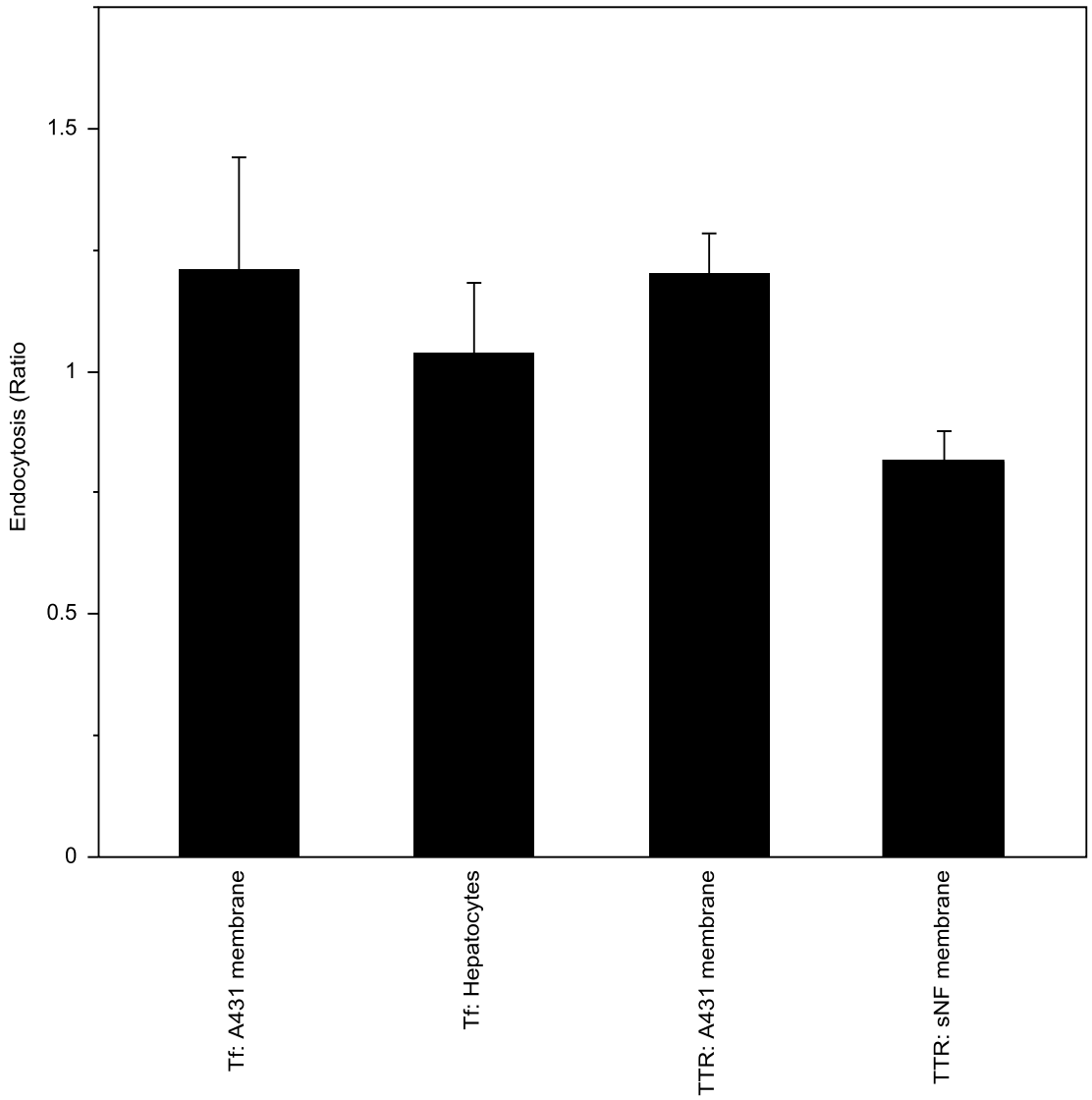
A comparison of apocynin effects on endocytosis of different ligands (Tf, TTR) in different cells or cell membrane preparations (hepatocytes, epidermoid, and Schwannoma lines) is shown in **Figure 3.8.4**. To facilitate comparisons, each bar represents a ratio of [proxidant + apocynin] / [prooxidant]. Comparison of ratios among the different cell/membrane types yielded a statistically significant difference between the epidermoid and Schwannoma lines in terms of TTR endocytosis ($p < 0.05$). Relative to controls without apocynin (only hydrogen peroxide), the hepatocytes and A431 cells/membranes were not significantly affected by apocynin ($p > 0.05$). The fact that three different A431 assays (the two shown in **Figure 3.8.4** and the one shown in **Table 3.3**), however, all yield [proxidant + apocynin] / [prooxidant] ratios > 1 (range 1.10 and 1.21) may be indicative of biological significance (related to membrane structure/function, as discussed further in the next Chapter) for apocynin in these peroxide-treated epidermoid cells.

Unexpectedly, the presence of apocynin (along with peroxide) in the assay with sNF membranes further decreased endocytosis of TTR (ratio below 1 in **Figure 3.8.4**), $p < 0.05$ for apocynin + hydrogen peroxide relative to hydrogen

peroxide alone. This effect was not observed for endocytosis of the same ligand, TTR, in the A431 membranes, and is considered further in the Discussion section.

Figure 3.8.4. Effect of apocynin on the hydrogen peroxide-induced inhibition of endocytosis.

Both different cell types/membranes (A431, sNF) and ligands (Tf, TTR) were tested. A statistically significant difference was observed for TTR endocytosis between A431 and sNF membranes ($p < 0.05$); *n* = 4-6 for each experimental condition. [apocynin] = 20 microM. Additional comparisons of statistical significance are described in the main text.

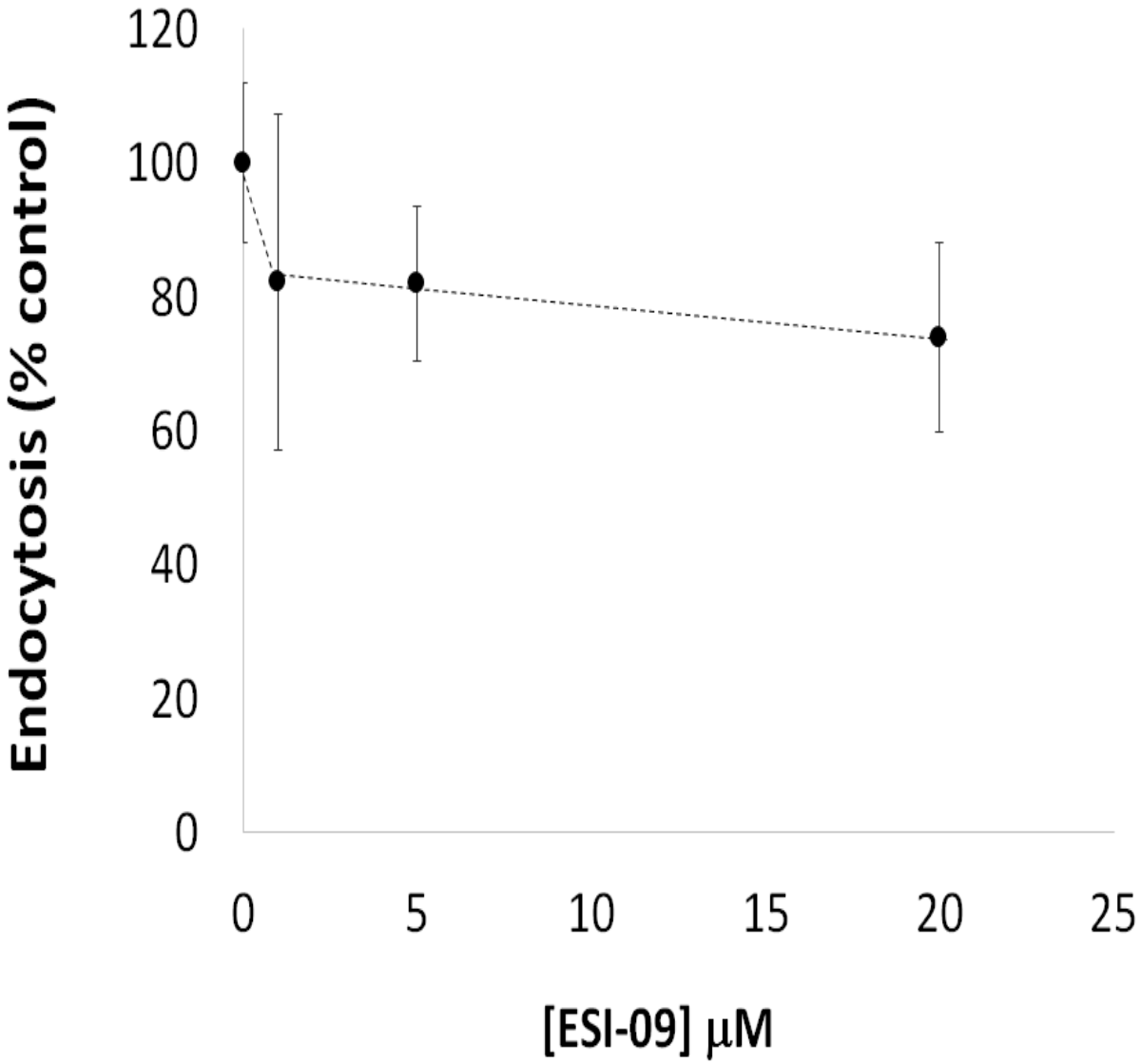


3.3.2. Effects of the EPAC inhibitor ESI-09 on endocytosis in liver cells

The ESI-09 compound, 3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-propionitrile, is an inhibitor of EPAC proteins (exchange protein directly activated by cAMP) (see related Discussion and references therein). This compound was tested for inhibition of clathrin-mediated endocytosis by examining Tf endocytosis after a 30 min pre-treatment. As shown in **Figure 3.9.1**, this pharmacological compound resulted in about 25% inhibition of Tf endocytosis, but only at the highest concentration tested (20 μM). Linear regression on the ESI-09 data showed no statistical significance ($p=0.108$). With a t-test to examine the difference between the means, the highest concentration of ESI-09 tested, 20 μM, was statistically significant compared to controls without the compound ($p<0.05$).

Figure 3.9.1. Effect of the synthetic EPAC inhibitor ESI-09 on the clathrin-mediated endocytosis of transferrin.

After a 30 min pre-treatment with the compound, endocytosis of Tf was analyzed in primary mouse liver cells. A statistically significant effect was observed at the highest concentration tested, 20 microM, relative to controls with only solvent (p=0.048, n =3); means +/- standard deviations.



Chapter 4. Discussion

4.1. Discussion of the effects of pro-oxidants and aging on endocytosis

4.1.1. Hydrogen peroxide and other pro-oxidants

To date there is reported evidence (References in Table 3.1) for the inhibitory effects of hydrogen peroxide, an ROS pro-oxidant, on the clathrin-mediated endocytic transport pathways involving transferrin and epidermal growth factor in two human cancer cell lines, HeLa (cervical) and A431 (epidermoid). In the current thesis project, additional evidence is provided for this pro-oxidant effect (cf. Table 3.1), as well as novel evidence for pro-oxidant-mediated inhibition in primary (non-transformed) mammalian cells, murine hepatocytes (Figures 3.1.1 and 3.1.2). Not only does hydrogen peroxide inhibit, the current results also

indicate that other pro-oxidants such as iron-ascorbate can decrease endocytic activity (Figures 3.1.1 and 3.2.1).

Interestingly, endocytosis in a reconstituted *in vitro* experimental system (based on semi-intact cell membrane preparations incubated with cytosol and ATP) can also be inhibited by hydrogen peroxide, but the inhibition is about 10-20% less than that observed in the same intact cells from which the membranes were derived (Figure 3.1.2). This result implies that the reconstituted *in vitro* system, while not exactly reproducing all the properties of the intact cell environment, can be useful in studies of endocytosis and in screening modulators of endocytic transport.

Relative to the primary hepatocytes, the human epidermoid cell line (A431) is more sensitive to hydrogen peroxide (Figure 3.1.2; and may also be more sensitive to an antioxidant as discussed further in the apocynin section). A possible explanation for this result is that the transformed line has greater redox instability, e.g., a lower antioxidant capacity. Other factors may contribute to the difference between the two cell types; for example, EGF receptor (EGFR) transactivation has been observed in response to hydrogen peroxide treatment in the EGFR-rich A431 cells (Smirnova et al., 2010). As suggested in Table 3.0, high peroxide (2.5 mM) concentrations may have a greater inhibitory effect on EGF relative to Tf internalization. Overall, the results indicate that the Tf CME pathway does not have the same sensitivity to inhibition by pro-oxidants in the

different cell types tested. A similar result on the differential sensitivity of Tf CME to an endocytic inhibitor, chlorpromazine, has been reported (Kalia et al., 2013).

As mentioned in the Introduction, there are over ten different endocytic pathways in cells, and probably others yet to be established. To date, the possible differential effects of pro-oxidants on different endocytic pathways were not well understood. In the current project, novel evidence is provided which shows that different endocytic pathways can respond differently to a given pro-oxidant (Figure 3.2.1 and 3.2.2). HRP uptake was used as an indicator of non-specific endocytosis, contributed to by multiple internalization pathways. With hydrogen peroxide treatment, HRP uptake was inhibited to a greater extent relative to Tf uptake in mouse hepatocytes (Figure 3.2.1). This result suggests that some of the pathways by which HRP is entering the cell are more sensitive to peroxide than transferrin's clathrin-mediated pathway. With iron-ascorbate as the prooxidant, however, the opposite result was observed for HRP and Tf. This result suggests different reactive species or different oxidative mechanisms are being induced by these two pro-oxidants. Although these two pro-oxidants may result in similar reactive oxygen species, there may be a difference in how the cells and endocytic machinery respond to the respective reactive mechanisms. Results with piscine hepatocytes (Figure 3.2.2) show a similar trend to that seen in murine hepatocytes (Figure 3.2.1), but the differences were not statistically significant.

4.1.2. agTTRs

Misfolded, aggregated TTR (agTTR) represents a pro-oxidative factor with potential pathological implications. agTTR is a pathological factor in neurodegenerative diseases such as senile systemic amyloidosis (SSA) and familial amyloidal polyneuropathy (FAP). agTTR was tested in the current study for possible effects on endocytosis; and it exhibited inhibitory activity relative to normal, soluble TTR in both mouse hepatocytes and human epidermoid carcinoma cells (Figure 3.3.1, Figure 3.3.2). These results are consistent with other research which shows that FAP is associated with oxidative damage and that agTTR leads to the increased release of hydrogen peroxide (Fong and Vieira, 2013; Ando et al., 1997; Sousa et al., 2001). Endocytosis and degradation of agTTR itself is likely to be important in moderating SSA and FAP disease progression; and fibroblasts have a major role in such clearance of agTTRs (Misumi et al., 2013). Perhaps moderating the agTTR-induced production of reactive chemical species with select antioxidants may increase the efficiency of such clearance by fibroblasts and impact disease progression.

4.1.3. Aging

There is some evidence that older cells have decreased efficiency of endocytic transport (examples and references in Table 3.2). The underlying mechanisms by which aging can affect such transport, however, are not well understood. One possibility is that increased oxidative stress in old tissues contributes to decreased endocytic transport efficiency. As indicated in this Table, previous *in vivo* studies have suggested an estimate endocytic deficiency of about 25% in lung, liver and kidney tissue from old animals (~26-28 month old mice) relative to young (~1-2 month old mice). In the current study, antioxidant capacity (inverse of oxidation) of the same old and young tissues was compared using a TMPD-based oxidation assay (Materials and Methods). Unexpectedly, there was little difference in the antioxidant capacity between old and young tissues (Figure 3.4.1), and no obvious indication of a higher capacity in the younger tissues. In fact, contrary to what was hypothesized, the means for old/young (O/Y) ratios of antioxidant capacity were all ~1 or >1 for the three tissues (Figure 3.4.2); while the O/Y estimated endocytosis efficiency ratios, as expected, were all < 1. At present a full explanation is lacking for these results on oxidation capacity of old vs young tissues; one possibility is that the assay used is not appropriate for assessing redox balance in these tissue extracts. Future studies are planned using a different method, a redox-sensitive electrical probe. The above results, however, do not necessarily rule out the possibility of greater oxidative damage

accumulation in the older tissues compared to the young ones (cf. Rodriguez-Biez et al., 2014; Sakul et al., 2013; Massudi et al., 2012). It will also be interesting to examine in future experiments whether select phytochemicals can moderate the inhibition of endocytosis in older tissues compared to younger tissues.

Table 4.1. Possible effects of aging on endocytic transport,

from Chen 2005; nd means 'no data.' The numbers refer to the following references: 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.Chen 2005, estimated endocytic efficiency; 6. Dini et al. 1996; 7. Haq and Szewczuk 1992; 8.Cessac et al. 1993, estimated endocytic efficiency; 9.Blanpied et al. 2003.

Cells/Tissues and Organisms	**Age comparisons** (in months unless noted otherwise)	**Age-related decrease in endocytosis** (ligand)
Kupfer cells (rat liver)	*nd* 6-12 vs. 22-36	35% (colloidal carbon)[1] 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]

....continued

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]

4.1.4. Vanadium

Vanadium is a trace element with a wide range of reported biological activities (reviewed in Crans et al., 2004). Vanadium is redox active (e.g., Garner et al., 1997; Crans et al., 2010), and it has been suggested that vanadium compounds may work synergistically with some antioxidants (Wang et al., 2010). Some of the reported physiological and pathological actions of vanadium are likely based on modulation of signal transduction (Barrio, 2010; Wang et al., 2010; Cuncic et al., 1999). Inhibition of phosphatases, and related increased phosphotyrosine, are found in some, but not all, cell types treated with vanadium compounds (Cuncic et al., 1999). Because of the well known relation between signal transduction (including that based on phosphotyrosine) and endocytosis, and because of its redox effects, vanadium was tested in the current study for potential modulation of endocytic transport. Two endocytosed ligands were compared in this part of the study, Tf and vitamin D-binding protein (VDBP). VDBP is a circulatory calciferol carrier that is endocytosed by a specific, but incompletely defined, clathrin-independent pathway; studies with chemical inhibitors suggest that this is likely to be a caveolar endocytic pathway.

Vanadate decreased the 10 min (37^{o}C) accumulation of both Tf and VDBP ligands to similar extents (Figure 3.5.1) relative to controls. When total binding of Tf and VDBP to the cells was assessed, however, DBP binding was about 30% lower relative to that of Tf. This result suggests that vanadate may lower the cell surface expression of VDBP receptors (although more detailed binding studies would be required to confirm). Overall the results suggest that vanadate may affect the distribution of internal versus cell surface receptors for both Tf and VDBP, with a potentially greater effect on the latter. A similar effect of Tf receptor redistribution by exposure to another metal ion, lead, has been reported (Quian and Morgan, 1990).

4.2. Discussion of the modulation of endocytosis by hormonal and growth factors

4.2.1. Epidermal growth factor

As with transferrin (Tf), epidermal growth factor (EGF) can be endocytosed through a CME pathway. Inhibition of the EGF endocytic pathway(s) is known to influence EGF-induced signal transduction (Omerovic et al., 2012 and references therein). Addition of EGF to cells results in ligand-induced endocytosis of the EGF receptor (EGFR), and initiation of intracellular signal transduction pathways through the receptor-tyrosine-kinase (RTK) activity of EGFR (Wang et al., 2005). Moreover, inhibitors of EGFR RTK activity have been reported to suppress ligand-stimulated endocytosis of EGFR (Nishimura et al. 2007). Do the signalling effects of EGF in cells modulate the uptake of other CME and non-CME ligands? This question was examined by analyzing the effects of EGF on the transport of ligands representing different endocytic pathways.

In terms of the CME of transferrin, the results (Figure 3.6.1) suggest that EGF signalling can have a moderate effect on this endocytic pathway, dependent on the length of time that the EGF is incubated with the cells. A 30 min pre-incubation decreases Tf uptake by about 20% relative to assays performed

without EGF pre-incubation (i.e., EGF is only present acutely during the 10 min endocytosis time; $p < 0.05$ for pre-incubation vs. no pre-incubation). This result suggests that EGF transport and signalling over a 30 min time period can interfere with clathrin-mediated transport events for other ligands. Although changes in gene transcription are unlikely to be involved over the 30 min, the difference relative to the 10 min treatment suggests a time-dependent signaling effect of EGF on endocytic transport. The inhibition of VDBP uptake, representative of a specific non-clathrin pathway, was not significantly affected by EGF pre-incubation ($p > 0.05$, Figure 3.6.2). This result suggests that EGF transport and signalling over 30 min does not impact this other endocytic pathway in the primary liver cells (but can impact non-CME in other cells, see below). HRP uptake, which is non-specific and includes all endocytic pathways that capture extracellular fluid, showed a trend towards a minor inhibition; but the difference to control was not statistically significant (Figure 3.6.2).

A previous study of acute-treatment EGF on endocytosis also reported a lack of effect of this growth factor (relative to controls without EGF) on the CME of Tf; but EGF had a stimulatory effect on a different, non-CME endocytic pathway (Sandvig and vanDeurs, 1990). As shown in Figure 3.6.3 EGF can stimulate the non-CME VDBP endocytosis in A431 cells to a much greater extent than in primary hepatocytes and relative to control. The current results suggest a differential response of epidermoid cells and hepatocytes in terms of EGF-modulated VDBP endocytosis. Overall, signalling pathways such as those of

EGF may affect endocytosis, for example, by affecting the cell surface/intracellular distribution ratio of the ligand receptor, or by other effects on the endocytic machinery.

4.2.2. Ethinyl estradiol

As mentioned in the Introduction, there is some but very limited work on the effects of steroid hormones on endocytosis. As with growth factors (EGF above), such work on steroid hormones may provide insight into poorly understood mechanisms of physiological regulation. In the current study, a synthetic estrogen, 17 alpha-ethinyl estradiol (EE) was examined for potential effects on endocytosis. EE was chosen because it has some reported effects on membrane structure and endosome acidification that could potentially affect endocytic transport (Rosario et al., 1988; Kitani et al., 1996; Goldsmith et al., 1982; Van Dyke et al., 1993).

The results for the effects of EE on transport (Figure 3.7.1) indicate a statistically significant inhibition of ligands representing two specific endocytic pathways (Tf, CME; and VDBP, non-CME; $p < 0.05$ in both cases relative to control without EE). The non-specific uptake of HRP was not significantly

affected by EE. A possible explanation for these results is that EE affects membrane structure such that some endocytic pathways are inhibited (Tf and VDBP); but others pathways are not affected, or may even be induced, by EE such that the general fluid-phase uptake of the cell (HRP) is not greatly affected. It will be interesting to evaluate the effect of EE in different cell types, such as breast cancer cells, in future experiments.

4.3. Modulation of endocytosis by phytochemicals and pharmacological factors

4.3.1. Apocynin

As mentioned in the Introduction, various phytochemicals and pharmacological agents can affect endocytic transport. Apocynin (1-(4-Hydroxy-3-methoxyphenyl)ethanone; Figure 3.8.1) is a phytochemical initially characterized from the Himalayan plant *Picrorhiza Kurroa*. It has been reported to have anti-inflammatory and antioxidant properties (Table 3.3 and references therein; Schreurs and Cipolla, 2014)). In the current study, apocynin was tested for its

potential to rescue inhibition of endocytic transport by pro-oxidants such as hydrogen peroxide.

When tested against the inhibition of endocytosis by hydrogen peroxide, apocynin did not rescue the transport in the primary liver cells (Figure 3.8.2). Other cell and membrane experimental systems were then analyzed for transport of Tf and another ligand transthyretin (TTR). TTR is a circulatory carrier protein that can transport thyroid hormones directly and vitamin A (retinol) indirectly through interaction with a vitamin A-carrier protein (RBP). There is controversy regarding the TTR endocytic pathway; but most studies suggest that it is not clathrin-mediated. As shown in Figure 3.8.3, the only statistically significant effect of apocynin was a further inhibition of transport in sNF cells (a human Schwannoma-like line; $p < 0.05$ relative to A431 membranes). Relative to the other cell/membrane sources, this unexpected (inhibitory) action of apocynin in sNF may be a result of a component that interacts antagonistically with apocynin resulting in enhanced pro-oxidant activity of either apocynin or of that component itself. The fact that TTR transport in A431 membranes was affected in the opposite way (ratio > 1 in Figure 3.8.3) suggests that the component postulated above is not present, or is present at lower levels, in A431 relative to sNF. Overall, these results suggest that factors in the Schwannoma cell interact with peroxide-apocynin to further inhibit a non-CME transport pathway. In this context, it has been reported that, relative to their normal Schwann cell counterparts,

there is an impairment in rates of membrane traffic in Schwannoma cells (Hennigan et al., 2013).

Because of the possible weak antioxidant activity of apocynin in these pro-oxidant-inhibited transport assays (first three bars of Figure 3.8.3), its antioxidant activity was directly tested in a peroxide-heme oxidation assay. As shown in Figure 3.8.4, no statistically significant antioxidant (nor pro-oxidant) activity was detected in this assay. In the context of the effects on endocytic transport discussed above, it will be of interest in future experiments to include sNF and A431 membranes in the oxidation assay. Although some previous studies show an antioxidant effect of apocynin, there is also current research which shows its potential for in vivo therapeutic effects in the absence of antioxidative properties (Khanicheh et al., 2013). The reason why some, but not other, *in vitro* oxidation assays with apocynin show antioxidant activity is likely due to different reagents or reagent concentrations. Widely varying antioxidant capacities for a given phytochemical (or phytochemical extract) in different assays have been reported from other studies (Nguyen et al., 2013, and references therein). Typically, there are many differences between experimental and physiological conditions, e.g., in terms of antioxidant metabolism and concentration levels.

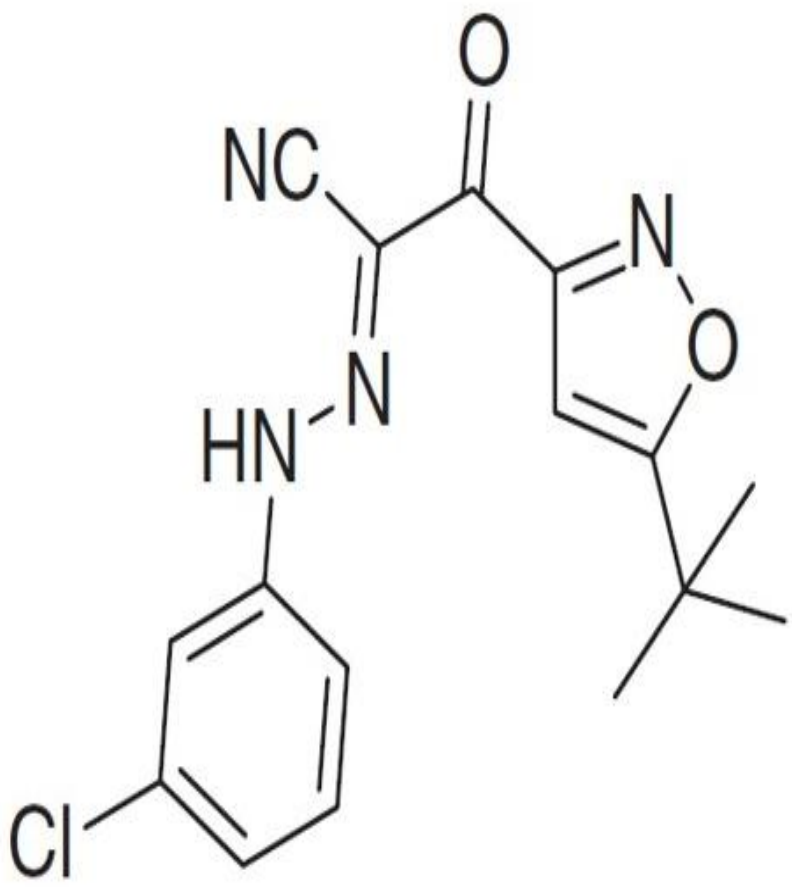
4.3.2. Cyclic EPAC inhibitor

The pharmacological compound ESI-09 (**Figure 4.1**) is an inhibitor of exchange protein directly activated by cAMP (Epac). It may prevent invasion of endothelial cells by rickettsiae, and decrease mortality in otherwise fatal rickettsiosis (Gong et al., 2013). Rickettsia is a genus of gram-negative bacteria that are obligate intracellular parasites.

Because cell invasion requires some form of endocytic transport, we hypothesized that this compound could inhibit one or more of the endocytic pathways being examined in this thesis project. Infections by some types of bacteria, perhaps even some of the rickettsiae, have previously been shown to require clathrin-mediated endocytosis (Bur et al., 2013; Bonazzi et al., 2012; reviewed by Veiga and Cossart, 2006). After a 30 min pre-treatment of cells with ESI-09, only the highest concentration tested (20 μM) showed some inhibitory activity. This concentration is about 5-15 fold higher than the IC50 concentrations for inhibitions of EPACs (Almahariq et al., 2013). More experiments are required to determine the IC50 for inhibition of Tf endocytosis. The compound should also be tested in different cell types for possible inhibition of CME and other endocytic pathways. If ESI-09 inhibits cell invasion by rickettsiae, and can do so at low micromolar (similar to IC50 for EPAC inhibition) or submicromolar concentrations, it may involve a non-CME pathway, perhaps a form of phagocytosis.

Figure 4.1. Chemical structure of ESI-09

3-(5-tert-butyl-isoxazol-3-yl)-2-[(3-chloro-phenyl)-hydrazono]-3-oxo-propionitrile, a compound that was tested for possible inhibition of endocytic transport. From Chen et al. (2013).



Chapter 5. Conclusions and questions for future research

5.1. Conclusions

5.1.1. Conclusions for the hypotheses

The following are conclusions in relation to each of the proposed *hypotheses* (from Chapter 1):

1. *Pro-oxidants inhibit endocytosis, and the extent of such inhibition differs with different pro-oxidants.* My results are in agreement with the hypothesis. Two different pro-oxidants, iron-ascorbate and hydrogen peroxide (at concentrations typically used in studies of oxidative stress) both can inhibit the CME pathway of Tf in the same cell type (murine hepatocytes).

2. *Different endocytic pathways in a given cell type exhibit different sensitivities to pro-oxidant inhibitors.* My results are in agreement with this hypothesis. In the same cell type (murine hepatocytes), the same concentration of hydrogen peroxide has greater effect on the non-specific, fluid-phase uptake of HRP compared to the specific CME of Tf. A different pro-oxidant, iron-ascorbate, has the opposite effect on the relative Tf and HRP uptake.

3. *Multiple endocytic pathways are known to be differentially active in different cell types. I hypothesize that inhibition of endocytosis by pro-oxidants also differs among different cell types.* My results are in agreement with this hypothesis. The same concentration of hydrogen peroxide has greater effect on the same endocytic pathway (Tf CME) in a line of human epidermoid carcinoma cells (A431) compared to primary murine hepatocytes.

4. *The antioxidant phytochemical apocynin can moderate the inhibition of endocytosis by pro-oxidants.* My results are not in agreement with this hypothesis. The presence of apocynin, at concentrations similar to or higher than those previously reported to have antioxidant effects, did not significantly increase endocytosis in the different cell types (or membrane preparations) tested.

5. *Decreased endocytic efficiency associated with aging differs in different cell types/tissues, and correlates inversely with the level of antioxidant capacity among those cells/tissues.* My results are not in agreement with this hypothesis. Based on the oxidation assay employed in my studies, there was no such inverse correlation between decreases in estimated endocytic efficiency and antioxidant capacity.

6. *Physiological factors such as steroid hormones and growth factors can modulate the efficiency of endocytic transport, and such modulation differs among different endocytic pathways.* My results are in agreement with this hypothesis. Primary murine hepatocytes pre-treated with EGF exhibited inhibition of Tf endocytosis (CME), but not VDBP endocytosis (non-CME). A different cell type, A431 human epidermoid cancer cells, exhibited and EGF-dependent stimulation of VDBP endocytosis. The synthetic estrogen EE, inhibited both the Tf and VDBP endocytic pathways in the same cell type, primary murine hepatocytes.

7. *A pharmacological compound that prevents infection of tissue by rickettsiae, can inhibit the clathrin-mediated endocytic transport of transferrin.* My results are in agreement with this hypothesis, but only at the higher concentration

tested, a concentration about 5-15 fold higher than the IC50 for inhibition of EPACs.

5.1.2. Other conclusions

The following are other major conclusions from my MSc research project:

A. Endocytic activity based on an established semi-intact cell membrane assay was also responsive to some of the modulators tested; e.g., inhibition of Tf sequestration by 0.5 mM hydrogen peroxide. My results provide more evidence for the validity of such assays.

B. Sequestration of ligand (i.e., endocytosis) by sNF membranes in the presence of hydrogen peroxide was further inhibited by the presence of the phytochemical apocynin. Because apocynin has exhibited antioxidant activity in some previously reported oxidation assays, I had expected a rescue of peroxide-inhibited transport.

C. Apocynin, up to 20 microM, does not exhibit antioxidant activity in a hydrogen-peroxide-heme-based oxidation assay.

D. An amyloidogenic (pathological) misfolded, aggregated protein (agTTR) inhibited endocytosis relative to the same, normally folded, soluble protein (TTR). Such inhibition by agTTR was observed in the two cell types tested, primary murine hepatocytes and a human epidermoid carcinoma line (A431).

5.2. Questions for future research

The following are questions of interest for future research in this field:

A. What is the basis for inhibition of endocytic transport by agTTR? If the basis is pro-oxidative effects, can the inhibition be rescued by antioxidants?

B. Are there other phytochemicals (apart from apocynin tested in the current study) that are potent at protecting endocytic activity against oxidative stress?

C. Does vanadate affect the internal vs. surface receptor distribution for VDBP alone, or does it also modulate receptors for other non-clathrin endocytic pathways?

D. Does oxidative stress affect other endocytic pathways such as phagocytosis?

E. How do different pro-oxidants such as hydrogen peroxide and iron (with fixed ratio of ascorbate) differ in their IC50 values for Tf endocytosis?

F. What are reasons why mouse liver cells appear to be more resistant to hydrogen peroxide compared to the other cell types tested?

G. Do some antioxidants increase the efficiency of agTTR clearance by fibroblasts and impact progression of TTR-amyloidogenic diseases?

H. Are rickettsiae bacteria endocytosed by CME, or by other endocytic pathways, or by multiple pathways?

I. As a basis for possible inhibition of endocytosis, does EE, or ESI-09, affect cell redox balance?

J. What is the basis of apocynin's additional inhibition of endocytosis in the presence of peroxide in sNF cells?

K. How might some metals such as vanadium and lead affect the cellular distributions of cell membrane receptors?

L. How might VDBP and TTR endocytic pathways differ?

M. What is the effect of selected phytochemicals such as apocynin on endocytic transport in old tissues compared with young tissues?

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