Fatty Acid Transport Protein Expression and Fatty Acid Transport across Human Brain Microvessel Endothelial Cells (HBMEC)

And

The Regulation of Cardiolipin Synthesis by Fatty Acid Transport Protein-1 (FATP-1)

by

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ABSTRACT

The blood-brain barrier (BBB), formed by the brain capillary endothelial cells, provides a protective barrier between the systemic blood and the extracellular environment of the central nervous system. Since most fatty acids in the brain enter from the blood, we examined the mechanism of permeability of various fatty acids across primary human brain microvessel endothelial cells (HBMEC). Cardiolipin (CL), a major mitochondrial phospholipid involved in energy metabolism in mammalian mitochondria, and fatty acid transport protein-1 (FATP-1) may regulate the intracellular level of fatty acyl-Coenzyme A's. Since fatty acids are required for oxidative phosphorylation via mitochondrial oxidation, we also examined the effect of altering FATP-1 levels on CL biosynthesis. The permeability of radiolabeled fatty acids was determined using confluent cells grown on Transwell® inserts in both the absence or presence of bovine serum albumin in the basolateral media, and following inhibition of various fatty acid transporters. The passage of [1-^{14}C]oleate across confluent HBMEC monolayers was significantly enhanced when fatty acid free albumin was present in the basolateral media. The presence of the non-specific fatty acid uptake inhibitor phloretin significantly decreased [1-^{14}C]oleate uptake by HBMEC and the subsequent release of [1-^{14}C]oleate into the basolateral medium. Knockdown of FATP-1, FATP-4, fatty acid translocase/CD36, or fatty acid binding protein 5 significantly decreased permeability of a number of radiolabeled fatty acids across the HBMEC monolayer from either apical or basolateral sides. The findings indicate that a fatty acid acceptor is a requirement for fatty acid transport across HBMEC monolayers. In addition, transport

of some fatty acids across HBMEC is, in part, a transcellular process mediated by fatty acid transport proteins. Next, HEK 293 cells were used as a model to determine the effect of altering FATP-1 levels on CL. HEK-293 mock- and FATP-1 siRNA-transfected cells or mock and FATP-1 expressing cells were incubated for 24 h with 0.1 mM oleate bound to albumin (1:1 molar ratio) then incubated for 24 h with 0.1 mM [1,3-^{3}H]glycerol and radioactivity incorporated into CL determined. FATP-1 siRNA-transfected cells exhibited reduced FATP-1 mRNA and increased incorporation of [1,3-^{3}H]glycerol into CL (2-fold, $p<0.05$) compared to controls indicating elevation in *de novo* CL biosynthesis. The reason for this was an increase in [1,3-^{3}H]glycerol uptake and increase in activity and mRNA expression of the CL biosynthetic enzymes. In contrast, expression of FATP-1 resulted a reduction in incorporation of [1,3-^{3}H]glycerol into CL (65%, $p<0.05$) indicating reduced CL synthesis. [1,3-^{3}H]Glycerol uptake was unaltered whereas activity of cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS) and CDS-2 mRNA expression were reduced in FATP-1 expressing cells compared to control. In addition, in vitro CDS activity was reduced by exogenous addition of oleoyl-Coenzyme A. The data indicate that CL *de novo* biosynthesis may be regulated by FATP-1 through CDS-2 expression in HEK 293 cells.

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ABBREVIATIONS

BBB	blood brain barrier
BSA	bovine serum albumin
CL	Cardiolipin
CNS	Central nervous system
CoA	coenzyme A
CPT	carnitine-palmitoyl transferase
CPT1	carnitine-palmitoyl transferase-1
CPT2	carnitine-palmitoyl transferase-2
CTP	cytidine triphosphate
DAG	diacylglycerol
DHA	docosahexaenoic acid
FABP	fatty acid binding protein
FABP5	fatty acid binding protein 5
FABP7	fatty acid binding protein 7
FAT/CD36	fatty acid translocase/CD36
FATP-1	fatty acid transport protein-1
FATP-2	fatty acid transport protein-1
FATP-3	fatty acid transport protein-1
FATP-4	fatty acid transport protein-1
FATP-5	fatty acid transport protein-1
FATP-6	fatty acid transport protein-1

FDX	Fluorescein isothiocynate labeled dextran
HBMEC	Human Brain Microvessel Endothelial Cell
HEK 293	Human Embryonic Kidney 293 cells
MAO	Monoamine oxidase
Ms	Mouse
PA	phosphatidic Acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGP	phosphatidylglcerol phosphate
PGE_2	prostaglandin E_2
PI	phosphatidylinositol
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidylserine
PUFA	polyunsaturated fatty acids
RXR	retinoid X receptor
SLC27A1	fatty acid transport protein-1
SM	sphingomylein
TEER	Transendothelial electrical resistance
ZO	Zonula occuldens

1 INTRODUCTION

1.1 Blood Brain Barrier

The blood-brain barrier (BBB) is the physical protective barrier between the cerebral blood vessels and parts of the central nervous system (CNS) (reviewed in Pardridge *et al*, 1975; Hawkins and Davis, 2005; Khan, 2005). It was first described by Paul Ehrlich in 1885 when acidic vital dyes injected into the blood stained all tissues in the body except for in the brain (Pardridge *et al*, 1975). Goldmann later confirmed the studies in 1913 and noted that dyes injected into the cerebrospinal fluid would only stain the brain and failed to stain the other tissues throughout the body, prompting him to coin the term 'blood-brain barrier' (Pardridge *et al*, 1975; Drewes, 1999; Hawkins and Davis, 2005). The BBB remained highly debated until the 1960s when Reese, Karnovsky and Brightman were able to visualize the endothelial cells lining the capillaries through electron microscopy (Drewes, 2001; Hawkins and Davis, 2005). The brain microvasculature is composed of three cell types that make up the BBB, the endothelial cell, the pericyte and the astrocyte foot processes (Pardridge, 1975; Hawkins and Davis, 2005). The endothelium and the pericyte share a common basement membrane and the astrocyte foot process interacts with 99% of the surface of this basement membrane (Cancilla *et al*, 1972; Kacem *et al*, 1998). In many tissues, the capillary endothelial cells are separated by small gaps, allowing solutes to pass freely into and out of the blood and into the surrounding tissues (Pries and Kuebler, 2006). In contrast, the brain capillaries are a continuous layer of endothelial cells with highly developed tight junctional complexes and a lack of fenestrations (**Figure 1**) (Reese and Karnovsky,

1967; Brightman and Reese, 1969; Fenstermacher *et al*, 1988; Hawkins and Davis, 2005; Pries and Kuebler, 2006). These tight junctions limit paracellular diffusion of solutes and macromolecules and force the BBB passage of substances to occur through transcellular routes (Pardridge *et al*, 1975; Pardridge and Mietus, 1980; Abbott, 2005; Seelig, 2007). The tight junctions of the brain endothelium provide one of the tightest monolayer of cells producing a transendothelial electrical resistance (TEER) of 1000 – 3000 ohm.cm^2 (Crone and Olesen, 1982).

Tight Junctional complex

The tight junctional complex between two adjacent brain endothelial cells is composed of a multitude of proteins that form both tight junctions and adherens junctions (Schulze and Firth, 1993; Kniesel and Wolburg, 2000; Wolburg and Lippoldt, 2002). Although both adherens junctions and tight junctions restrict the paracellular transport across the BBB, it is primarily the role of the tight junctions that provides the low paracellular permeability and high TEER across the BBB (Romero *et al*, 2003; Hawkins and Davis, 2005). Tight junctions are composed of three main proteins; junctional adhesion molecule (JAM-1), occludins and claudins (Furuse *et al*, 1993; Citi and Cordenonsi, 1998; Furuse *et al*, 1998; Del Maschio *et al*, 1999). JAM-1, a single transmembrane protein, provides the initial attachment between adjacent cell membranes through homophilic interactions of the extracellular domains (Dejana *et al*, 2000). Occludins and Claudins interact between two adjacent endothelial cells and form noncovalent bonds through their extracellular loop domains (Furuse *et al*, 1993; Heiskala *et al*, 2001). Claudins are hypothesized to provide the primary seal for the tight junctions and

occludins help to support the seal (Kubota *et al*, 1999; Hawkins and Davis, 2005). Furthermore, several accessory proteins such as ZO-1, -2 and -3, located in the cytoplasm, associate with tight junctions by providing a crosslink to cytoskeletal structures such as actin (Vorbrodt and Dobrogowska, 2003). Tight junctions also provide asymmetric distribution of membrane components, such as transport proteins, by separating the apical and basolateral cell membranes (Hawkins and Davis, 2005).

BBB function

The BBB provides a physical selective barrier between the CNS and the peripheral circulatory system. The BBB is a facilitator of transport of certain substances due to the presence of tight junctions, which force substances to move transcellularly and due to the presence of specific transport mechanisms on both the luminal and abluminal membranes (Hawkins and Davis, 2005; Abbott *et al*, 2006). The BBB is a protective barrier against potential harmful substances by excluding and effluxing waste products back into the periphery (Begley and Brightman, 2003; Hawkins and Davis, 2005; Abbott *et al*, 2006). The endothelial cells of the BBB and the astrocytes that envelop the cerebral capillaries also provide a metabolic barrier for the brain against permeable substances (Pardridge *et al*, 1975; el-Bacha and Minn, 1999; Abbott *et al*, 2006). Both intracellular and extracellular enzymes contribute to the metabolic barrier and protect the brain from potentially harmful compounds by inactivating them into polar compounds, thus preventing them from further crossing into the brain (Abbott *et al*, 2006). This is demonstrated through the action of monoamine oxidase (MAO) on the neurotransmitter dopamine. Dopamine released from neurons is readily taken up by the

endothelium where MAO converts it to its inactive metabolite (3,4-Dihydroxyphenylacetic acid) and is effluxed into the circulating plasma resulting in minimal systemic effects (Drewes, 1999). Thus the endothelial cells of the BBB maintain proper brain function by creating a highly regulated interface between the blood and the extracellular fluid of the brain.

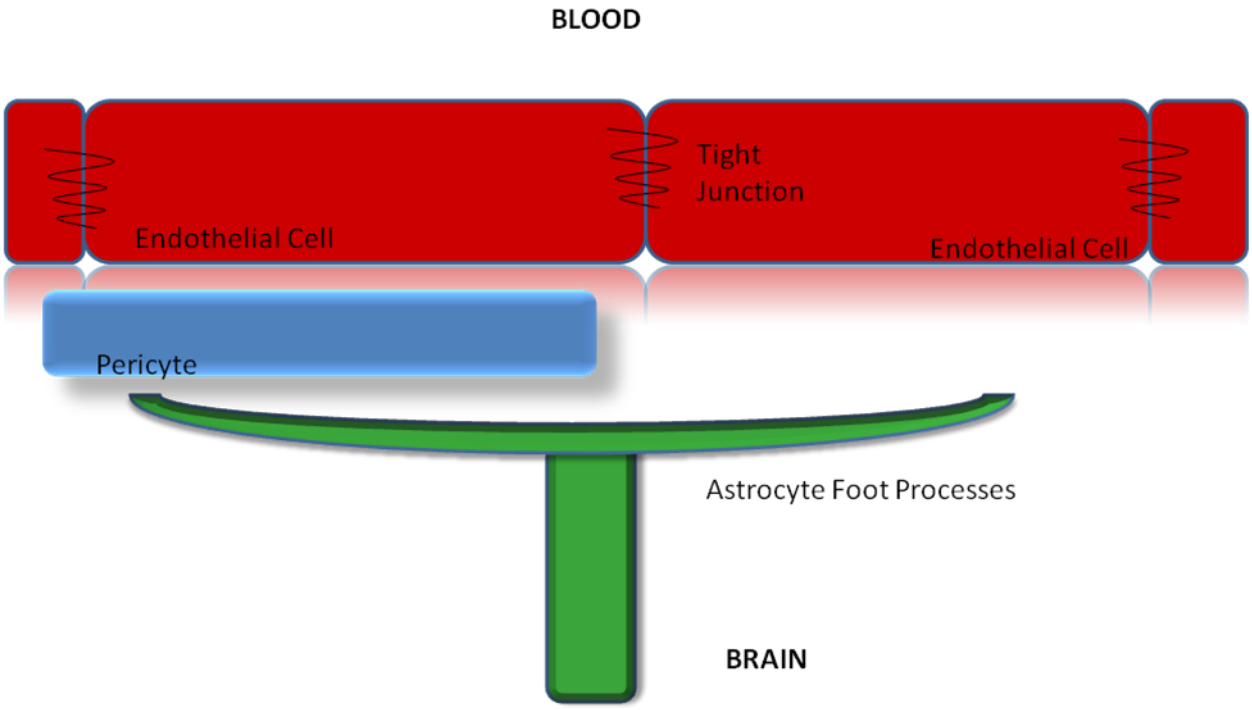

Figure 1

Blood-brain barrier. Brain microvessel endothelial cells surrounded by pericytes and astrocyte foot processes, making up the microvasculature unit of the BBB. The presence of tight junctions holds the endothelium closely together forming a tight monolayer and severely restricting paracellular passage.

1.2 Fatty Acids

Fatty acids are important substrates and have many functions for a variety of tissues. Fatty acids are an important energy source for cells via mitochondrial β-oxidation. Fatty acids have a higher energy density than glucose and are stored as triacylglycerol in the cytosol of many tissues (Abumrad *et* al, 1999; Schaffer, 2002). The heart tissue is able to receive up to 70 % of its energy needs through fatty acid oxidation (Bing *et al*, 1954). Long-chain fatty acids can either be synthesized from *de* novo synthesis, hydrolysis of triglycerides or taken up from exogenous sources (Schaffer, 2002). However, only a small amount of fatty acids are derived from hydrolysis of intracellular triacylglycerol stores (Brouns and van der Vusse, 1998). Most cells other than adipocytes and hepatocytes cannot synthesize or store fatty acids in the amounts required for their oxidative energy needs and therefore are dependent upon fatty acid uptake from the blood (Abumrad *et al*, 1999). Fatty acids are also important components of membranes; they are the precursors for the biosynthesis of phospholipids and sphingolipids that make up the membrane lipids (Yamashita *et al*, 1997). The acyl chain composition of the membrane lipids plays an important role and determines the overall structure and function of the membrane (Jackson *et al*, 1995; Mundy, 1995; Schaffer, 2002). Unesterfied fatty acids are important intracellular regulators of gene expression. They serve as ligands for transcription factors such as HNF4 (Hepatocyte nuclear factor-4) and the PPAR (peroxisome-proliferator-activated receoptors) family (Bernlohr *et al*, 1997; Distal *et al*, 1992). Fatty acids also induce the aP2 gene involved in adipose cell

differentiation (Amri *et al*, 1991). Fatty acids can directly regulate ion channel activation in certain cells (Ordway *et al*, 1991). Arachidonic acid and other fatty acids activate potassium ion channels in smooth muscle cells (Ordway *et al*, 1989) and calcium channels in cardiac sarcolemmal vesicles (Philipson and Ward, 1985). Fatty acids are precursors of lipid-signaling molecules, eicosanoids and leukotrienes. Long-chain fatty acids trigger the release of insulin by β-cells through the activation of a G-protein-coupled receptor, GPR40 (Steneberg *et al*, 2005).

Fatty acids are absorbed by enterocytes in the small intestine and are reformed into triglycerides and packaged into chylomicrons. Chylomicrons are then released by enterocytes and enter the lymphatic system and then the bloodstream. Lipoprotein-lipase, produced by heart, liver, adipose tissue and other tissues releases the fatty acids from the lipoproteins and subsequently the majority binds to albumin. Fatty acid-albumin complexes are transported in the bloodstream and are taken up by tissues in the body (Pohl *et al*, 2004). Fatty acids enter into cells and are esterfied or are transported to the mitochondria for β-oxidation.

Fatty acids are essential for both the developing and adult mammalian brain (Dhopeshwarkar and Mead, 1973; Simopoulos, 1989; Edmond *et al*, 1998; Rapoport *et al*, 2001). Lipids make up roughly 50% of the brain's dry weight making it the organ with the second highest lipid content next to adipose tissue (Morell, 1996; Watkins *et al*, 2001). In the developing rat brain, the synthesis and rapid accumulation of long-

chain saturated and unsaturated fatty acids is necessary for accelerated growth and development of the brain as well as the onset of myelination (Cuzner and Davison, 1968; Edmond *et al*, 1991, 1998). Docosahexaenoic acid (DHA, 22:6n-3) is the major structural omega-3 (n-3) polyunsaturated fatty acid (PUFA) in the cell membranes of the brain and retina (Innis, 1991**)**. DHA incorporation into these tissues is done primarily during the perinatal brain growth spurt (Innis, 1991). Evidence suggests that inadequate supplies of DHA and other n-3 PUFA during this period results in reduced membrane accumulation of DHA and subsequently impaired learning capabilities, neurotransmission process and visual function (Aïd *et al*, 2003; Alessandri *et al*, 2004; Chalon, 2006). Autism, dyslexia as well as a number of other neurodevelopment disorders has been associated with altered fatty acid metabolism during development of the brain (Horrobin, 1999; Bell *et al*, 2000; Clayton *et al*, 2008). In the adult mammalian brain, altered fatty acid metabolism has been linked to the onset of certain neurological disorders such as, depression, bipolar disorder and Alzheimer's disease (Söderberg *et al*, 1991; Hibbeln, 1998; Stoll *et al*, 1999; Pawels and Volterrani, 2008; Rapoport, 2008; Lee *et al*, 2010). Fatty acids have been linked in the treatment of a variety of neurodegenerative diseases and in the general neuroprotection of the brain (Bordet *et al*, 2006; Rao *et al*, 2008). Even fatty acid transport proteins have become a promising therapeutic target to modulate lipid movement and metabolism (Glatz *et al*, 2010). Fatty acids are natural ligands of PPAR, which regulate lipid and lipoprotein metabolism (Keller *et al*, 1993). There are three isoforms of PPARs (α, β/δ, γ) and all are expressed in cerebral blood vessels, neurons and astrocytes (Moreno *et al*, 2004).

PPAR activation by fatty acids has been implicated in the regulation of neuronal cell death during ischemia, neurodegenerative or inflammatory cerebral diseases (Bordet *et al*, 2006). PPARγ agonists modulate inflammatory responses and prevent endotoxin-induced neuronal death (Kim *et al*, 2002). PPARα and PPARγ agonists also inhibit Aβ (amyloid β-peptide) deposition in neuritic plaques found in the hippocampus and cortex of Alzheimer mouse models (Combs *et al*, 2001; Heneka *et al*, 2005).

Glucose is the main energy source for the adult brain but fatty acids may be used indirectly during starvation, diabetes or in the neonatal period, when glucose supply is low (Drewes *et al*, 1973; Hawkins *et al*, 1971). Fatty acids are catabolized to ketone bodies, acetoactate and β-hydroxybutyrate, which can be transported across the BBB through a carrier-mediated transport mechanism (Crone and Gjedde, 1972). The switch in fuel consumption by the brain from a combination of glucose and ketone bodies to complete glucose is done in the normal development of the brain (Cremer, 1982). n-3 PUFA may also modulate glucose transport across brain microvessel endothelial cells and its utilization by the brain (Pifferi *et al*, 2007). n-3 PUFA deficiency decreased glucose transporter, GLUT1 protein content by 23% and n-3 PUFA supplementation increased GLUT1 protein content by 37%, thus modulating glucose utilization by the brain (Pifferi *et al*, 2007).

The brain is capable of synthesizing only a few non-essential fatty acids; therefore both essential fatty acids, and even some nonessential fatty acids, must enter the brain from

the blood (Dhopeshwarkar and Mead, 1973). The main source of arachidonic acid in the brain is from the blood, as linoleic acid that enters into the brain is primarily beta-oxidized and thus is a negligible source of arachidonic acid in rat brain (DeMar *et al*, 2006). Fatty acids do not readily cross the BBB as components of phospholipids or triglycerides (Dhopeshwarkar and Mead, 1973; Pardridge *et al*, 1975; Spector, 1988). However, palmitic acid and arachidonic acid were shown to enter in rat brain microvessels from the plasma (Williams *et al*, 1997). Fatty acid saturation and chain length appear to play an important role in the transport of fatty acids across the BBB (Spector, 1988). The mechanism of transport of fatty acids into the brain is relatively unknown.

1.3 Fatty Acid Transport

The mechanism of fatty acid import into cells has been a source of constant debate. There are two opposing views on fatty acid transport across cell membranes and import into cells. Fatty acids, like other hydrophobic molecules, can cross the lipid bilayers by passive diffusion, using a 'flip-flop' mechanism independent of proteins (Hamilton *et al*, 2001; Hamiton *et al*, 2002). The other view suggests that fatty acids enter cells with the help of a specific protein-mediated transport mechanism (Abumrad *et al*, 1998; Abumrad *et al*, 1999; Schwenk *et al*, 2010). The transport of fatty acids across cells involves five steps which are consistent with both diffusional and protein-mediated components (Black and DiRusso, 2003). Fatty acids as part of micelles or albumin complexes are unable to cross cell membranes, therefore step one of fatty acid transport

involves the dissociation of fatty acids from albumin to free fatty acid and make it available to the cell membrane (Black and DiRusso, 2003). Next, free fatty acid is delivered to the membrane (step 2) and is transferred across the exoplasmic side of the membrane to the cytoplasmic side of the membrane in step 3 (Black and DiRusso, 2003). Step 4 involves the movement of fatty acids out of the membrane followed by intracellular lipid metabolism (step 5). These steps could occur spontaneously by diffusional components or could occur through protein mediation.

Diffusion

In the passive diffusion model, fatty acids dissociate from albumin and bind to the outer leaflet of the plasma membrane. This free diffusion is highly dependent upon the lipophilicity and the molecular size of the blood-borne substance (Levin, 1980). Short chain and medium chain fatty acids, twelve carbons or less, have high permeability coefficients and easily cross the lipid bilayer (Kamp *et al*, 2003). Long-chain fatty acids (> 12 carbons) are less soluble, thus are more problematic in fatty acid transmembrane movement than shorter fatty acids (Hamilton, 1999). Long-chain fatty acids need to be in the nonionized form for rapid diffusion across the plasma membrane to occur (Kamp *et al*, 2003). The movement of long-chain fatty acids in their ionized form across plasma membranes is very slow (Gutknecht, 1988). Thus, once long-chain fatty acids bind to the outer leaflet, they quickly reach ionization equilibrium and the non-ionized form of fatty acids move across the membrane more rapidly than the ionized form (Hamilton, 1999). These steps of rapid fatty acid transport and transmembrane movement (flip-flop) is performed through a protein-independent transport mechanism

with no expenditure of cellular energy (Kamp *et al*, 1993; Kamp *et al*, 1995; Kamp and Hamilton, 2006). Several studies have supported this flip-flop mechanism using unilammelar phosphatidylcholine (PC) membrane vesicles and determining the transport of nonionized long-chain fatty acids (Schaffer, 2002). The addition of free long-chain fatty acids to these vesicles resulted in rapid flip-flop of the fatty acids occurring within milliseconds (Kamp *et al*, 1995). Subsequent studies demonstrated similar rates when fatty acids were complexed with BSA in red blood cell ghosts (Kleinfeld *et al*, 1998). However, is the passive flip-flop diffusion model quick enough to fulfill the fatty acid requirement of cells with a high long-chain fatty acid metabolism? This remains the most controversial aspect of the passive diffusion model (Hamilton *et al*, 1998).

Transport proteins

In contrast, in the protein mediated transport model, selective transport of fatty acids is accomplished by specific protein transporters expressed on the cell membrane (reviewed in Abumrad *et al*, 1998; Frohnert and Bernlohr, 2000; Hui and Bernlohr, 1997; Schaffer, 2002; Storch and Corsico 2008). Genetic studies performed in *E. coli* identified two genes involved in long-chain fatty acid transport, *fadL* and *fadD* (reviewed in Black and DiRusso, 1994). Kinetic studies of fatty acid transport demonstrate a maximal and linear transport for 15 s in isolated adipocytes (Stump *et al*, 2001). This transport was saturable at low fatty acid:BSA ratios, consistent with the physiological range (0.74 fatty acid/albumin ratio) (Richieri and Kleinfeld, 1995) and is specific to long-chain fatty acids (Abumrad *et al*, 1984). Cell types that undergo high

levels of fatty acid metabolism are able to transport fatty acids at a higher rate than those cell types with lower lipid metabolism, suggesting a highly regulated fatty acid transport protein mechanism (Dutta-Roy, 2000; Ibrahimi *et al*, 1999). Studies have demonstrated that roughly 90% of long-chain fatty acid uptake by adipocytes is done by a saturable mechanism (Stump *et al*, 2001). Other tissues, such as the liver, intestine and the heart, demonstrate a specific and saturable uptake pathway of long-chain fatty acids as well (Abumrad *et al*, 1998; Gore and Hoinard, 1993; Sorrentino *et al*, 1988; Stremmel, 1988; Stremmel, 1989a; Stremmel, 1989b). There are three classes of fatty acid transport proteins that are responsible for protein-mediated fatty acid transport. Fatty acid transport protein (FATP), fatty acid translocase/CD36 (FAT/CD36) and fatty acid binding protein (FABP) are discussed below.

Fatty acid transport into cells becomes more complicated as they traverse into the brain because of the presence of the BBB. Tight junctions severely restrict the passage of many compounds into the brain. Thus, the major pathway across the BBB and into the brain is by transcellular transport across both the luminal and abluminal membranes of the endothelial cells (Drewes, 1999). Fatty acids must move across the luminal (apical) and abluminal (basolateral) membrane of the BBB endothelial cells, and then across the plasma membrane of the neural cells (Kamp, 1993, 1995; Watkins *et al*, 2001). Is it feasible, that this movement is quick enough to supply the brain with their fatty acid requirement without fatty acid transporters?

1.4 Fatty Acid Transport Proteins

There are currently four protein families that have been implicated in fatty acid transport. These include the fatty acid transport proteins 1-6 (FATP1-6), fatty acid translocase/CD 36 (FAT/CD36), fatty acid binding proteins (FABP), both plasma membrane fatty acid binding protein (FABPpm) and cytosolic fatty acid binding proteins ($FABP_c$) and caveolin-1 (Stremmel *et al*, 1985 ; Schaffer and Lodish, 1994; Schaffer, 1996, 2002; Harmon and Abumrad, 1993; Hui and Bernlohr, 1997; Trigatti *et al*, 1999; Doege and Stahl, 2005).

Fatty acid transport protein (FATP)

Fatty acid transport protein is a 71 kDa protein, identified by Schaffer and Lodish in 1994, located on the plasma membrane as well as some intracellular membranes. There are six isoforms of FATP identified in human cells, FATP-1-6 (Hirsch *et al*, 1998). Its 311 amino acid sequence is highly conserved among family members (Faergeman *et al*, 1997; Hirsch *et al*, 1998). Human FATPs have also been discovered to have great homology to FATP in a number of species such as in mice, *Drosophila melanogaster*, *Saccharomyces cerevisiae*, along with other invertebrates (Berger *et al*, 1998; Hirsch *et al*, 1998).

FATP expression is tissue specific. FATP-1 is predominately expressed in adipose tissue (Schaffer and Lodish, 1994) but is also found in heart, skeletal and brain tissue

(Binnert *et al*, 2000; Fitscher *et al*, 1998; Hirsch *et al*, 1998). FATP-2 is expressed almost exclusively in liver and the kidney cortex (Hirsch *et al*, 1998). FATP-3 is more broadly expressed (Hirsch *et al*, 1998). It is more highly expressed in the lungs where uptake of long-chain fatty acids is used to help generate pulmonary surfactant (Jobe, 1979; Stahl *et al*, 2001). FATP-4 is the lone FATP expressed in the small intestine (Stahl *et al*, 1999). It is responsible for the absorption of dietary lipids (Stahl *et al*, 1999). FATP-4 along with FATP-1 are the predominant FATPs located in the brain (Fitscher *et al*, 1998). FATP-5 is exclusively found in the liver (Hirsch *et al*, 1998) and FATP-6 is the predominate FATP expressed in the heart (Gimeno *et al*, 2003; Watkins *et al*, 1999).

FATP is composed of at least one transmembrane domain with a number of membrane associated domains (Lewis *et al*, 2001). The amino terminus is located on the extracellular side and the carboxy terminus is on the cytosolic side of the membrane (Lewis *et al*, 2001). FATP has specificity to long-chain fatty acids, with no detectable uptake of fatty acids shorter than 10 carbon atoms (Schaffer and Lodish, 1994). FATP appears to have no specificity towards the saturation of fatty acids, as both palmitate and oleate uptake is enhanced with FATP-4 expression (Schaffer and Lodish, 1994; Stahl *et al*, 1999).

FATP has been identified as a bifunctional protein, coupling fatty acid import into cells with a long chain acyl-CoA synthetase (Coe *et al*, 1999; Hall *et al*, 2003). Studies of

FATP-1 identified two functional motifs, motif 1 is the ATP binding site involved in acyl-CoA synthetase activity and motif2 is the fatty acid binding domain (Coe *et al*, 1999; Watkins *et al*, 1998). FATP-1 facilitates the movement of fatty acids into cells and their activation for metabolism, observed when overexpression of FATP-1 increased rate of fatty acid influx and triacylglycerol synthesis in HEK 293 cells (Hatch *et al*, 2002). Overexpression of FATP-2 and FATP-4 have also demonstrated increased acyl-CoA synthetase activity in COS1 cells (Hermann *et al*, 2001; Uchiyama *et al*, 1996). FATP mRNA expression is tissue specific (Hirsch *et al*, 1998). Human FATP genes are regulated by insulin, peroxisome proliferator-activated receptor (PPAR) and retinoid X receptor (RXR) agonists (Martin *et al*, 1997; Martin *et al*, 1998; Martin *et al*, 2000). However, tissues where FATP-1 mRNA is highly expressed, such as the heart, PPAR and RXR agonists have no effect on the expression, FATP-1 is thought to be constitutively expressed in these tissues (Martin *et al*, 1998; Martin *et al*, 2000).

Fatty acid translocase/CD36 (FAT/CD36)

FAT/CD36 originally referred to as FAT (fatty acid translocase) is an 88 kDa integral membrane glycoprotein identified and isolated through the irreversible inhibition of fatty acid uptake by sulfo-succinimidyl oleic acid (SSO) (Harmon and Abumrad, 1993). The cDNA for the FAT protein encodes for 85% homology to human CD36, a thrombospondin-binding protein (Abumrad *et al*, 1993). FAT/CD36 has a hairpin topology defined by two transmembrane domains (Tao *et al*, 1996). Both the N-terminus and C-terminus are located in the cytoplasm (Tao *et al*, 1996) and the

extracellular domain is heavily glycoslyated (Abumrad *et al*, 1993; Jochen *et al*, 1995). An extracellular segment of FAT/CD36 contains homology to the fatty acid binding domains found on the heart subtype FABP (Baillie *et al*, 1996; Banaszak *et al*, 1994). FAT/CD36 is expressed on the membranes of a variety of different cells, such as adipocytes, platelets, macrophages and endothelial cells of the microvasculature (Abumrad *et al*, 2005). Thus FAT/CD36 has a number of functions and roles in atherogenesis, inflammation, platelet function and lipid transport and metabolism (Abumrad *et al*, 2005).

FAT/CD36 has a very important role in fatty acid uptake by cells and their metabolism. FAT/CD36 has a high affinity for long-chain fatty acids (Abumrad *et al*, 1993). Overexpression of FAT/CD36 results in an increased rate of fatty acid uptake (Ibrahimi *et al*, 1999) whereas FAT/CD36 null mice demonstrate a reduced fatty acid uptake (Coburn *et al*, 2000; Febbraio *et al*, 1999). FAT/CD36 is also a receptor for lipoproteins; on macrophages FAT/CD36 functions as a receptor for oxidized low-density lipoproteins (oxLDL) **(**Greenwalt *et al*, 1992; Febbraio *et al*, 2001**)**. FAT/CD36 has also been shown to have affinity for very long-chain fatty acids, as demonstrated in enterocytes with CD36-deficient mice (Drover *et al*, 2008). The exact mechanism of fatty acid transport by FAT/CD36 is not known. It is thought that FAT/CD36 is not a true transporter, rather a mediator to fatty acid transport. It mediates fatty acid dissociation from albumin, followed by the flip-flop across the phospholipid bilayer and into the cytosol (Abumrad *et al*, 2000). More recent studies demonstrate a strong

relationship between FAT/CD36 and lipid rafts. It is suggested that in order for fatty acid transport to occur, FAT/CD36 must be associated with lipids rafts on the plasma membrane (Ehehalt *et al*, 2008).

FAT/CD36 tissue distribution is highly dependent upon the high metabolic activity for long-chain fatty acids within the tissues (Abumrad *et al*, 1993). Tissues such as adipose tissue, muscle and intestines have high expression of CD36 whereas tissues such as the liver have low expression (Abumrad *et al*, 1993). CD36 is also expressed in brain tissue, in which it has high expression in endothelial cells of the brain microvasculature (Husemann *et al*, 2002). Regulation of FAT/CD36 is mediated by long-chain fatty acids and insulin (Luiken *et al*, 2002*a*,*b*; Sfeir *et al*, 1997). Long-chain fatty acids and insulin stimulate the translocation of FAT/CD36 from intracellular depots to the plasma membrane (Luiken *et al*, 2002*a*,*b*; Sfeir *et al*, 1997). PPARs do not appear to regulate the expression of FAT/CD36 (Bonen *et al*, 2004).

Fatty acid binding protein (FABP)

Fatty acid binding proteins (FABP) are made up of two different protein families, found in different cellular locations but both function in fatty acid transport and lipid metabolism. Membrane-associated fatty acid binding protein ($FABP_{PM}$) is a 40 kDa protein that is associated with the plasma membrane (Stremmel *et al*, 1985). $FABP_{PM}$ is expressed in a variety of tissues including, the liver, adipose tissue, the heart and the intestine (Schwieterman *et al*, 1989; Stremmel, 1988; 1989a; 1989b). $FABP_{PM}$ not only

binds fatty acids with high affinity but also cholesterol, lysophosphatidylcholine and monoacylglycerol (Stremmel *et al*, 1985; Stremmel and Diede, 1989). Expression of $FABP_{PM}$ is induced during differentiation of 3T3 fibroblasts which consequently results in increased fatty acid uptake rates (Zhou *et al*, 1992). $FABP_{PM}$ is also regulated when fatty acid utilization is enhanced, such as during fasting (Turcotte *et al*, 1997) and during endurance training (Kiens *et al*, 1997). $FABP_{PM}$ is also regulated during disease states, such as in diabetic Zucker and obese rats, both mRNA of $FABP_{PM}$ and fatty acid uptake rates are elevated (Berk *et al*, 1997).

Evidence for $FABP_{PM}$ involvement in fatty acid transport is provided by a number of studies in which $FABP_{PM}$ is inhibited or overexpressed and is related to fatty acid uptake rates. Antibodies against $FABP_{PM}$ inhibited fatty acid uptake by roughly 50% in hepatocytes, myocytes and adipocytes (Berk *et al*, 1990; Zhou *et al*, 1992). Expression of $FABP_{PM}$ in 3T3 fibroblasts increased fatty acid uptake through a saturable process, which was inhibited by antibodies against $FABP_{PM}$ (Zhou *et al*, 1992). The mechanism of fatty acid uptake by $FABP_{PM}$ is still relatively unclear. It has been proposed that its role is to bind free fatty acids and transfer them to fatty acid transport proteins, which in turn transfer them across the plasma membrane and into the cells (Dutta-Roy, 2000).

The other FABP family is composed of a number of small, 14-15 kDa, cytosolic proteins (Ockner *et al*, 1972). FABP expression is tissue specific with nine different subtypes having been identified thus far (reviewed in Glatz and van der Vusse, 1996).

FABPs such as FABP3 and FABP1 are expressed primarily in heart and liver, respectively, however they are also expressed in other tissues, such as stomach, intestine, brain and adipose tissue (Veerkamp and Maatman, 1995). Other FABP are exclusively expressed in one tissue, FABP7 in the brain, FABP4 in adipose tissue, FABP5 in epidermal cells, and FABP2 in the interestine (reviewed in Glatz and van der Vusse, 1996; Veerkamp and Maatman, 1995). High expression of one or more FABP in tissues is strongly associated with high rates of fatty acid uptake and metabolism (Storch and McDermott, 2009).

FABP structure is similar among the different subtypes with ten antiparallel β-strands and two parallel α-helices (Zimmerman *et al*, 2001). These cytosolic FABPs bind long-chain fatty acids with high affinity (Richieri *et al*, 1994). The different FABP subtypes have different affinity and specificity for fatty acids and other ligands (Glatz and van der Vusse, 1996; Veerkamp and Maatman, 1995; Zimmerman *et al*, 2001). Unsaturated fatty acids appear to bind to FABP with a higher affinity than saturated fatty acids; oleic acid and arachidonic acid bind to FABP with higher affinity than palmitic acid (Richieri *et al*, 1994, Zimmerman *et al*, 2001). This may be explained by the lower aqueous solubility of saturated fatty acids compared to the polyunsaturated fatty acids (Richieri *et al*, 1994). The carboxyl group of fatty acids interacts with the FABP in its binding cavity capped by the two α-helices, creating a 'lid' enclosing the bound fatty acid (Glatz and van der Vusse, 1996; Storch and McDermott, 2009). Once bound, fatty acids are released by FABP by two different mechanisms, by aqueous

phase diffusion between two membranes and by collision transfer, in which there is physical interaction between FABP and membranes (Storch and Thumser, 2000).

The primary function of FABP is fatty acid uptake, translocation and lipid metabolism. FABP3 knock-out mice models demonstrate decreased fatty acid uptake and metabolism by isolated cardiac myocytes compared to the wild-type (Schaap *et al*, 1999). Docosahexaenoic acid (DHA) uptake by FABP7 null mice was significantly decreased and its accumulation in the brain was reduced (Owada *et al*, 2006). Disruption of FABP4 in mice altered fatty acid metabolism by decreasing lypolysis in isolated adipocytes however the disruption of the FABP4 gene did not decrease fatty acid uptake (Coe *et al*, 1999). Thus the different FABP subtypes appear to have different functions based on tissue expression. A number of different FABP are expressed in different cell types within tissues and may confer different specificity and function, including in the brain (Zimmerman *et al*, 2001). There are four FABP subtypes found in nervous tissue, FABP3, FABP5, FABP6 and FABP7 (Owada *et al*, 1996). FABP6 is found only in the peripheral nervous system whereas FABP3 and FABP5 are found in neurons and glial cells in the central nervous system of both pre- and perinatal brain (Owada *et al*, 1996; Veerkamp and Zimmerman, 2001). FABP5 is also highly expressed in the endothelial cells of the microvasculature (Masouyé *et al*, 1997). FABP7 shows a strong specificity to polyunsaturated fatty acids (Xu *et al*, 1996). FABP7 does not bind palmitic acid but binds to DHA with high affinity, attributable to the fact that DHA enrichment in the brain is important for its development (Xu *et al*,

1996). FABPs are implicated in a number of other functions, such as, modulation of intracellular fatty acid levels, signal transduction and gene transcription (Glatz and van der Vusse, 1996; Zimmerman *et al*, 2001).

Caveolin-1

Caveolae are intracellular invaginations of the plasma membrane that are formed from lipid raft domains (Pike *et al*, 2002; Razani *et al*, 2002; McFarland *et al*, 2004; Su and Abumrad, 2009). Lipid rafts are high in sphingoliopids and cholesterol (Simons and van Meer, 1988). There are three isoforms of the structural proteins that make up the caveolae, caveolin-1, caveolin-2 and caveolin-3 (Razani *et al*, 2002). Each caveolin is a 22 kDa integral membrane hairpin-like protein that faces the cytosol (Razani *et al*, 2002; Thompson *et al*, 2010). Caveolin-1 and caveolin-2 are expressed throughout the body, however, caveolin-3 is muscle-specific (Su and Abumrad, 2009). These caveolae microdomains have a number of functions, including cholesterol transport, endocytosis and potocytosis (Sowa *et al*, 2001; McFarland *et al*, 2004). Caveolin-1 has also been linked to long-chain fatty acid uptake in certain cells (Stremmel *et al*, 2001; Pohl *et al*, 2002; Ehehalt *et al*, 2006). Trigatti *et al* (1999) first demonstrated that caveolin-1 is able to bind to fatty acids. However, it is still unclear whether caveolin-1 is directly involved in fatty acid uptake or indirectly through an association with FAT/CD36.

Several studies have demonstrated that caveolin-1 acts directly in the uptake of long-chain fatty acids by providing a vesicle for these fatty acids to enter into cells

(Stremmel *et al*, 2001; Pohl *et al*, 2002; Pohl *et al*, 2004). These fatty acid filled vesicles stained positive for caveolin-1 in HepG2 cells (Stremmel *et al*, 2001). Inhibition of lipid rafts by β- cyclodextran or overexpression of mutant caveolin significantly reduced the uptake of long-chain fatty acids in 3T3-L1 adipocytes (Pohl *et al*, 2004). HEK293 do not express fatty acid transport proteins nor do they express detectable levels of caveolin-1. Thus, when studies demonstrated that the expression of caveolin-1 in HEK293 cells resulted in a two-fold increase in fatty acid uptake by these cells, it provided strong evidence for the direct involvement of caveolin-1 in the uptake of fatty acids (Meshulam *et al*, 2006). The proposed mechanism of this uptake is the binding of fatty acids to caveolin-1 in the caveolae domains. Budding of this caveolar domain from the plasma membrane occurs to form vesicles in the cytosol, these vesicles are then delivered to subcellular organelles (Thompson *et al*, 2010).

Caveolin-1 may also act indirectly in the uptake of fatty acids by controlling the localization of CD36 to the plasma membrane (Ring *et al*, 2006; Thompson *et al*, 2010). CD36 is abundant in lipid rafts and appears to interact functionally with caveolin-1 (Ehehalt *et al*, 2008; Su and Abumrad, 2009). Caveolin-1 expression is essential for the stabilization and localization of CD36 to the plasma membrane (Ring *et al*, 2006). CD36 modulates the function of caveolin-1 through phosphorylation by the Src kinases that associate with the C-terminus of CD36 (Labrecque *et al*, 2004; Eyre *et al*, 2006; Eyre *et al*, 2008; Su and Abumrad, 2009). Caveolin-1 knockout mice studies demonstrated the link between caveolin-1 and CD36 and fatty acid uptake. These

knockout mice presented with complete loss of caveolae as well as a loss in the expression of CD36 in the plasma membrane which resulted in a reduction in fatty acid uptake into the cells (Ring *et al*, 2006). This reduction in fatty acid uptake could be restored when caveolin-1 was expressed and subsequently CD36 was localized to the plasma membrane (Ring *et al*, 2006).

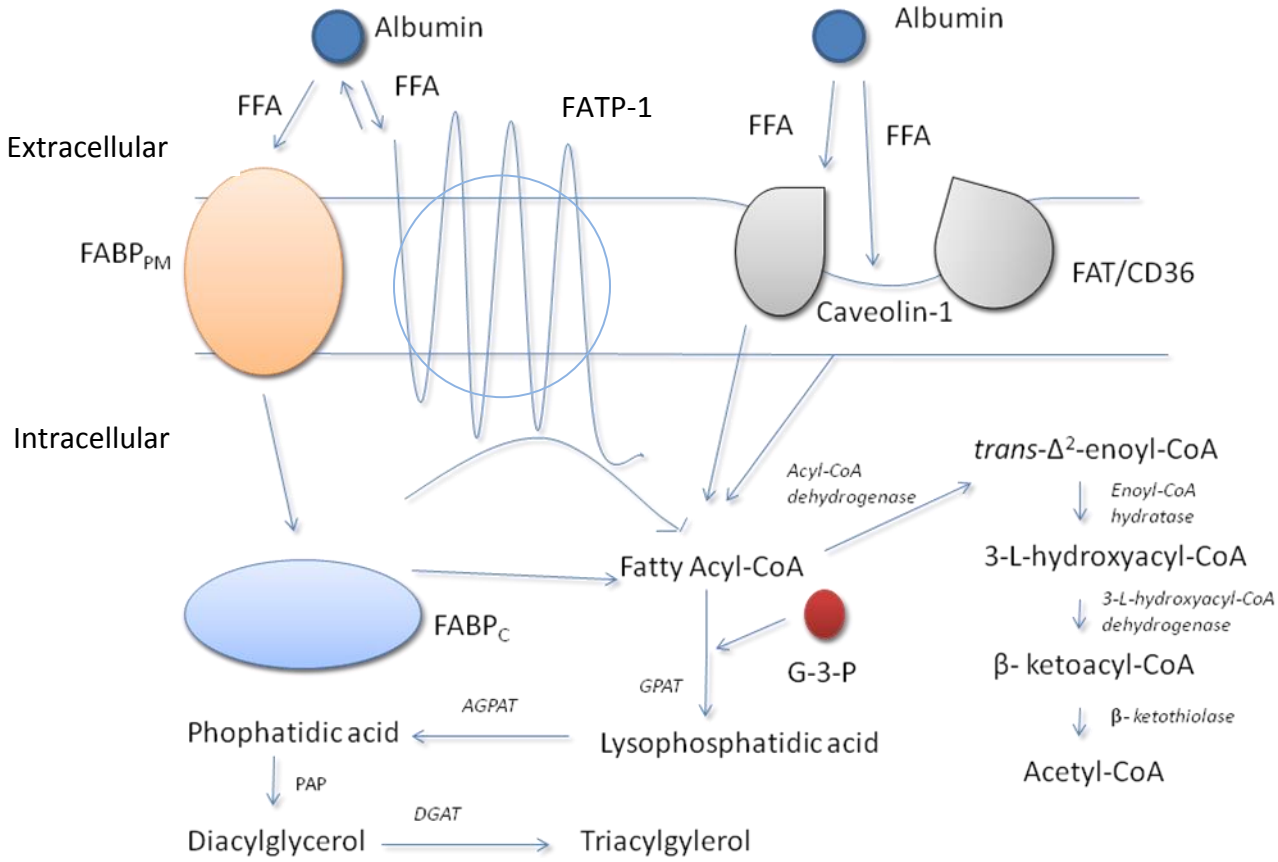

Figure 2

Fatty acid transport proteins and subsequent β-oxidation and triacylglycerol biosynthesis from fatty acyl-CoA. G-3-P, glycerol-3 –phosphate; GPAT, glycerol-3-phosphate acyltranserase; AGPAT, acylglycerophosphate acyltransferase; PAP, phosphatidate phosphatase; DGAT, diacylglycerol acyltransferase.

1.5 Fatty acid uptake into brain

Fatty acid transport into the brain is made more complicated by the BBB. Total fatty acid concentration in the blood is roughly 0.4 mM - 0.5 mM (Pardridge *et al*, 1975). However, because 99% of circulating fatty acids are bound to albumin, the amount of fatty acid available to the brain and other tissues is quite small (Pardridge *et al*, 1975). At physiological levels, the concentration of unbound 'free' fatty acids is 7.5 nM (Richieri and Kleinfeld, 1995). Proteins are unable to cross the BBB, thus fatty acids must dissociate from the plasma protein and transfer to the BBB membrane in order for them to enter into the brain (Saunders, 1977). As stated earlier, if free diffusion were the sole mechanism for transport, polar substances would be expected to penetrate the BBB very slowly. This has been shown not to be the case in the transport of certain polar compounds, suggesting the presence of transport proteins (Pardridge *et al*, 1975). Drewes suggests that current evidence supports simple diffusion of fatty acids across the BBB but also realizes that fatty acid transport proteins may function in an additive fashion to the diffusion of fatty acids (Drewes, 1999).

It has been well established that fatty acids, as components of triglycerides or phospholipids, are not transported across the rat BBB (Dhopeshwarkar *et al*, 1972; Dhopeshwarkar and Mead, 1973; Pardridge *et al*, 1979). Transport of cholesterol and fatty acids across the BBB is more controversial. There is evidence that supports that dietary labeled cholesterol is able to cross the BBB (Serougne *et al*, 1976), however,

there is also evidence to support that labeled cholesterol does not cross the BBB and enter the fetal or adult rat brain (Morris and Chaikoff, 1961). It is unclear if the vascular compartment of the brain was separated from the rest of brain, resulting in the contribution of residual radioactivity from the blood in the brain to the overall brain radioactivity. Early studies performed by Dhopeshwarkar and Mead (1973) in which cholesterol and fatty acid uptake by the brain was low, provided evidence that the BBB was impermeable to these substances (Pardridge *et al*, 1975). Recent studies have now shown that nearly 100% of cholesterol in the brain is synthesized *in situ* and only negligible amounts of cholesterol is taken up by lipoproteins from the peripheral blood (Björkhem and Meaney, 2004; Dietschy and Turnley, 2004). During the brain growth spurt and prelude to myelination, deuterated fatty acids did not accumulate in the brain as compared to other tissues, prompting researchers to conclude that the brain synthesizes saturated and monounsaturated fatty acids and does not depend on an exogenous fatty acid supply during the increased fatty acid requirement (Edmond *et al*, 1998). Thus, it is commonly believed that the brain is able to synthesize *in situ* saturated and monounsaturated fatty acids and consequentially must transport essential fatty acids from the blood by highly specific mechanisms (Edmond *et al*, 1998; Edmond, 2001). This was demonstrated when perdeuterated saturated and monounsaturated fatty acids, such as palmitic acid or oleic acid, were fed to rat pups and the labeled fatty acids accumulated in all other organs except the brain (Edmond *et al*, 1998). However, labeled linoleic acid and other polyunsaturated fatty acids were found to accumulate in the brain thus at least partially relying on uptake from the blood and transport across the

BBB (Edmond *et al*, 1998).

Fatty acid transport proteins are variably expressed in the brain with some regions conferring a higher expression than in other regions of the brain. Fatty acid transport proteins also have different expression profiles in the developing and mature brain (Kurtz *et al*, 1994; Owada *et al*, 1996; Utsunomiya *et al*, 1997). These transport proteins have a discrete spatio-temporal gene expression (Owada *et al*, 1996; Utsunomiya *et al*, 1997). FATP, FABP7 and FABP5 are highly expressed in the ventricular germinal zone, composed of both glial and neuronal precursor cells in developing rat brains, however, their expression is notably absent in the cerebellar external granule cell layers, which is solely a neuronal precursor germinal zone (Kurtz *et al*, 1994; Utsunomiya *et al*, 1997). This suggests that FATP, FABP7 and FABP5 are localized in the glial cells of immature rat brains (Utsunomiya *et al*, 1997). FATP and FABP3 mRNA is highly expressed in neurons, localized in the cerebellar Purkinje and granule cell layers in the mature rat brain (Utsunomiya *et al*, 1997).

The lipid composition of the brain is high in polyunsaturated fatty acids (PUFA), roughly 30% of the total lipid content of the brain are PUFAs (Spector, 2001). The mechanism by which the brain maintains this high PUFA composition is still debated. There are two proposed models recently reviewed by Chen *et al* (2008). One of the models suggests that lipoproteins are the main source of PUFAs for the brain. These lipoproteins are taken up by the endothelium through receptor mediated transport and

the esterified fatty acids can then be hydrolyzed within the cell and then further transported across into the brain to be metabolized and incorporated into phospholipids. The other model suggests that lipoprotein lipase which is expressed on the endothelium can cleave the triacylglyceol component of the lipoproteins and the resulting fatty acids can then be transported across either by protein mediated transport or passive diffusion into the brain.

1.6 Fatty acid transport into mitochondria

As previously stated, once long-chain fatty acids enter cells they are either esterfied or are transported to the mitochondria to undergo β-oxidation. Historically, the carnitine-palmitoyl transferase (CPT) system was thought to be the only mechanism involved in transport of fatty acids into the mitochondria (McGarry and Brown, 1997; Kerner and Hoppel, 2000). This system consists of carnitine-palmitoyl transferase-1 (CPT1), acylcarnitine translocase and carnitine-palmitoyl transferase-2 (CPT2) (McGarry and Brown, 1997). The mitochondrial inner membrane is impermeable to acyl-CoAs, therefore CPT1 catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines, which are then translocated across the mitochondrial inner membrane by acylcarnitine translocase (Kerner and Hoppel, 2000; Campbell *et al*, 2004; Sebastián *et al*, 2009). In the final step acylcarnitine is reconverted back to acyl-CoAs by CPT2 in order for β-oxidation to occur (Kerner and Hoppel, 2000; Campbell *et al*, 2004; Sebastián *et al*, 2009). There are three isoforms of CPT1 that are expressed in different

tissues, CPT1A is expressed in the liver, CPT1B is expressed in muscle and CPT1C is expressed in the brain (Esser *et al*, 1993; Yamazaki *et al*, 1995; Price *et al*, 2002) This system is regulated by malonyl-CoA and provides a signal to the cell on the availability of lipids for energy production (McGarry and Brown, 1997). Regulation of CPT1 alone cannot explain the changes in mitochondrial fatty acid import seen when there is an increased energy demand by the cell (Odland *et al*, 1996; Dean *et al*, 2000).

FAT/CD36 was shown to be present in the mitochondrial membrane and having a potential role in the mitochondrial import of fatty acids (Campbell *et al*, 2004, Holloway *et al*, 2009). FAT/CD36 and CPT1 are co localized on the outer mitochondrial membrane (Campbell *et al*, 2004). The exact mechanism of action of FAT/CD36 on the mitochondrial membrane is not well known but it may be acting as an acceptor of long-chain fatty acids, bringing them to be activated by a long chain acyl CoA synthetase, which in turn are then converted to acylcarnitines by CPT1 (Campbell *et al*, 2004). FAT/CD36 KO mice demonstrated a normal basal mitochondrial fatty acid oxidation, suggesting FAT/CD36 does not play an essential role in mitochondrial fatty acid import (King *et al*, 2007). However, several studies suggest that FAT/CD36 may only be important in mitochondrial fatty acid import during times of increased energy demands, such as during exercise or muscle contraction (Ibrahimi *et al*, 1999; Bezaire *et al*, 2006; Schenk and Horowitz *et al*, 2006).

More recently, FATP-1 has also been shown to be localized in the mitochondria and

may potentially play a role in mitochondrial fatty acid import (Sebastián *et al*, 2009). Overexpression of both FATP-1 and CPT1 in mitochondrial fractions produced an addictive effect on mitochondrial fatty acid oxidation, suggesting that FATP-1 collaborates with CPT1 to import fatty acids into the mitochondria (Sebastián *et al*, 2009). FATP-1 and CPT1 physically interact in the mitochondria as both proteins coimmunoprecipitated in the mitochondrial fractions (Sebastián *et al*, 2009). The exact mechanism of the interaction and collaboration between FATP-1 and CPT1 has yet to be elucidated; however, both FATP-1 and FAT/CD36 appear to play important roles in mitochondrial oxidation of fatty acids. Thus, alterations in fatty acid transport proteins, FATP-1 and FAT/CD36 may cause alterations in the metabolism of phospholipids, including the highly important mitochondrial phospholipid, cardiolipin.

1.7 Cardiolipin

Cardiolipin (CL) is a polyglycerolphospholipid found in and synthesized exclusively in the mitochondria of mammalian tissues (reviewed in Daum, 1986; Hatch, 2004; Hoch, 1992; Hostetler, 1982). CL is localized to both the inner and outer mitochondrial membrane and within intermembrane contact sites. CL plays an important role in many mitochondria mediated functions. CL is required for the activation of a number of key mitochondrial enzymes, such as cytochrome c oxidase and other proteins involved in the electron transport chain, "gluing" the respiratory chain together (Daum, 1986; Hatch, 2004; Hoch, 1992; Hostetler, 1982; Zhang *et al*, 2002). Loss of CL leads to a

reduction in mitochondrial respiration (Kiebish *et al*, 2008). In addition, studies have implicated CL loss in the regulation of mitochondrial-mediated apoptosis and Barth Syndrome (reviewed in Chicco and Sparagna, 2007; Hauff and Hatch, 2006; Hootkeper and Vaz, 2008; Li *et al*, 2007; Ott *et al*, 2007; Schlame and Ren, 2006). Thus, the appropriate content of CL is an important requirement for activation of enzymes involved in mitochondrial respiration and in the control of programmed cell death.

1.8 Cardiolipin Biosynthesis Pathway

In mammalian tissues CL is synthesized *de novo* by the cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol (CDP-DG) pathway (**Figure 3**) (Hatch, 1994). The first step is catalyzed by cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol synthetase (CDS) (Kiyasu *et al*, 1963). CDS catalyzes the conversion of phosphatidic acid (PA) to CDP-DG. In the heart and various mammalian cell lines CDS was shown to be a rate-limiting step of CL biosynthesis (reviewed in Hatch, 2004). Two CDS enzymes, CDS-1 and CDS-2, have been cloned and characterized in human, mouse, rat and pig (Halford *et al*, 1998; Heacock *et al*, 1996; Lykidis *et al*, 1997; Mercade *et al*, 2007; Saito *et al*, 1997; Volta *et al*, 1999; Weeks *et al*, 1997). In the second step of the CDP-DG pathway CDP-DG is condensed with *sn*-glycerol-3-phosphate to form phosphatidylglycerol (PG) catalyzed by PG phosphate synthase (PGPS) and this is followed by rapid dephosphorylation to PG (Kiyasu *et al*, 1963). In the final step, PG condenses with another CDP-DG molecule to form CL and this is catalyzed by cardiolipin synthase (CLS) (Hostetler *et al*, 1971). The gene encoding human CLS (hCLS-1) was recently identified and

characterized (Chen *et al*, 2006; Houtkooper *et al*, 2006; Lu *et al*, 2006).

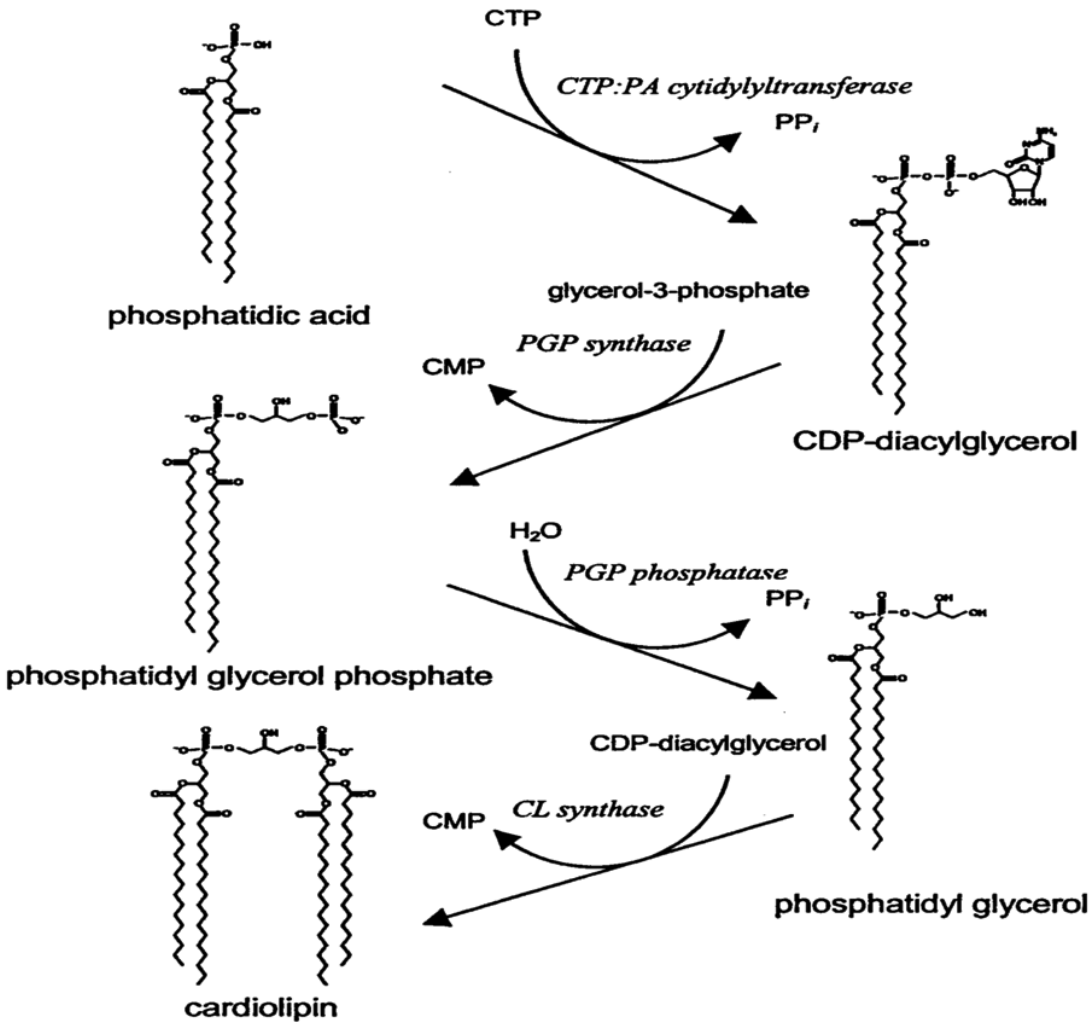

Figure 3

CDP-DG *de novo* biosynthetic pathway of CL. Copyright Hatch, 2004

1.9 Objectives

Fatty acids are major components of glycerophospholipids and are required for energy production via beta-oxidation (reviewed in van der Vusse *et al*, 2000). Fatty acids are taken up into cells either by diffusion due to their solubility in the membrane or by proteins which facilitate their transport (reviewed in Schaffer and Lodish, 1994; Hirsch *et al*, 1998; van der Vusse *et al*, 2000; Pohl *et al*, 2004). Currently three protein families have been implicated in fatty transport, fatty acid translocase/CD 36, plasma membrane fatty acid binding protein and fatty acid transport protein (FATP). Five members of the FATP family have been described in murine cells and a sixth identified from analysis of the human genome (Gimeno, 2007). FATP-1 plays a key role in regulating fatty acid transport in mammalian cells. FATP-1 is a bifunctional protein with both transport and long chain acyl-Coenzyme A synthetase activity (Hall *et al*, 2003).

Since essential and some nonessential fatty acids must enter the brain via the blood we examined the factors influencing fatty acid uptake and cellular processing in brain capillary endothelial cells. The focus of the present study was to identify and characterize the transcellular and paracellular routes for fatty acid transport across an *in vitro* BBB model. The results of these studies demonstrate that fatty acid passage across HBMEC is a transcellular process mediated, in part, by fatty acid transport proteins and is enhanced by the presence of a protein acceptor. Fatty acid transport proteins, FATP-1 and FAT/CD36, are associated with the mitochondrial membrane. Since fatty acid

import into cells and the mitochondria plays a key role in fatty acid oxidation and CL is required for oxidative phosphorylation, we examined whether altering the level of fatty acid transport protein in HEK 293 cells could regulate CL biosynthesis. We show that knock down of FATP-1 stimulates and expression of FATP-1 inhibits *de novo* CL synthesis.

2 MATERIALS AND METHODS

2.1 Materials

[^{14}C]oleic acid and [^{14}C]linoleic acid were obtained from PerkinElmer (Boston, Massachusetts, USA). [^{14}C]butyric acid, [^{14}C]myristic acid and [^{14}C]lignoceric acid were obtained from American Radiolabelled Chemicals, Inc. (St. Louis, Missouri, USA). [^{3}H]palmitic acid and [^{3}H]arachidonic acid were obtained from Amersham Pharmacia Biotech (Baie d'Urfe, Québec, Canada). [^{14}C]Glycerol-3-phosphate, [5-^{3}H]cytidine-5'-triphophate, and [1,3-^{3}H]glycerol and were obtained from either Dupont, Mississauga, Ontario, or Amersham, Oakville, Ontario, Canada. Phosphatidyl[^{14}C]glycerol was synthesized from [^{14}C]glycerol-3-phosphate as described previously (Hatch and McClarty, 1996). Primary human brain microvessel endothelial cells (HBMEC) obtained from normal human brain cortex tissue, cell culture media, and cell attachment factors were purchased from Cell Systems Corporation (Kirkland, Washington, USA) Polycarbonate Transwell® inserts (0.4 mm; 24 mm diameter) -were purchased from Corning Life Sciences, Wilkes-Barre, Pennsylvania, USA. Previously frozen human brain grey and white matter tissue samples were obtained from autopsies performed on two individuals <18 years age who died of non-neurological causes and whose brains were removed 16-20 hours after death. Autopsy consent included unrestricted permission for research purposes. Tissue samples were anonymized and handled with appropriate ethical considerations.. Human Embryonic Kidney (HEK) 293 cells were obtained from American Type Culture Collection. Dulbecco's modified

Eagle's medium (DMEM), fetal bovine serum (FBS) and Trizol reagent were obtained from Canadian Life Technologies (GIBCO), Burlington, Ontario, Canada. C8 HEK 293 cells expressing FATP-1 were a generous gift from Dr. David A. Bernlohr, University of Minnesota. Lipid standards were obtained from Serdary Research Laboratories, Englewood Cliffs, New Jersey, USA. Thin layer chromatographic plates (silica gel G, 0.25 mm thickness) were obtained from Fisher Scientific, Winnipeg, Manitoba, Canada. Ecolite scintillant was obtained from ICN Biochemicals, Montreal, Quebec, Canada. Fluorescein Dextran was obtained from Invitrogen, Burlington, Ontario, Canada. Phloretin and bovine serum albumin (BSA) were obtained from Sigma Chemical Company, St. Louis, MO, USA. Primers were designed and synthesized from Invitrogen, Burlington, Ontario, Canada. Flexitube siRNA and HiPerFect Reagent were obtained from Qiagen, Cambridge, Massachusetts, USA. Primary antibodies; SLC27A1, SLC27A4, FABP5 and CD36 and secondary antibody; Mouse IgG were obtained from Abcam, Cambridge, MA, USA. All other biochemicals were certified ACS grade and obtained from either Fisher Scientific, Winnipeg, Manitoba, Canada or Sigma Chemical Company, St. Louis, MO., USA.

2.2 Culturing of HBMEC

Immediately upon receipt, cells were cultured in a T25 flask until approximately 80-90% confluency and then passaged (1:3 split ratio) in T75 flasks. From the T75 flasks HBMEC were plated onto transwell plates (70,000 cells/cm^2) and permeability

experiments performed after reaching confluency (typically 3-4 days). Prior to plating, the Transwell® permeable inserts were coated with attachment factor (Cell Systems Corporation, Kirkland, Washington, USA). The HBMEC were maintained on CS-C complete medium with 50µg/ml CS-C growth factor™ (acidic fibroblast growth factor (aFGF) and porcine heparin), ciprofloxacin and 10% fetal bovine serum (FBS). Cells were incubated at 37°C in 5.0% CO_2 and media was replaced every two days until 50% confluence was reached then every day until cells were 100% confluent. Cells used in the current studies were passage 5 or lower and remained viable for the duration of the experiments as assessed by Trypan blue exclusion.

2.3 Culturing, radiolabeling and harvesting of HEK 293 cells

HEK 293 cells were transfected with FATP-1 subcloned into pcDNA3.0 (C8) or transformed with plasmid control vector (mock) as previously described (Hatch *et al*, 2002). Cell lines were maintained on DMEM with 10% FBS and incubated at 37°C in 5.0% CO_2. Cells expressing FATP-1 were selected with the addition of 400 ug/ml geneticin. In radiolabeling experiments, mock and C8 cell lines were incubated with 0.1mM oleic acid bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H]glycerol (10 µCi/dish) for 24 h. Subsequent to incubation the medium was removed and the cells washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS). The PBS was removed and 2 ml of methanol:water (1:1 v/v) was added. The cells were harvested using a rubber policeman into test tubes. The mixture was

vortexed, and a 50 µl aliquot was taken for protein determination and a 10 µl or 25 µl aliquot taken for determination of total radioactivity. 2 ml of chloroform was added to the tubes followed by 0.5 ml 0.9% NaCl to initiate phase separation. The tubes were vortexed and then centrifuged at 2,000 x g for 10 minutes (bench top centrifuge), the aqueous phase was removed and 2 ml theoretical upper phase (48 ml methanol, 47 ml 0.9% NaCl, 3 ml chloroform) was added to wash the organic phase. The tubes were vortexed and centrifuged as described above and the aqueous phase removed. The organic phase was dried under a stream of N_2 gas and resuspended in 50 µl of chloroform:methanol (2:1 v/v). A 40 µl aliquot of organic phase was placed onto a thin-layer plate along with phospholipid standards and phospholipids were separated by two-dimensional thin-layer chromatography and radioactivity incorporated into phospholipids determined as described (Hatch and McClarty, 1996). For determination of phospholipid pool sizes, in some experiments, a 40 µl aliquot of organic phase was placed onto a thin-layer plate in the absence of phospholipid standards and phospholipids were separated by two-dimensional thin-layer chromatography as pool size determined as described (Rouser *et al*, 1970).

2.4 Permeability studies

Confluent HBMEC monolayers were incubated apically with 0.1mM radiolabeled fatty acids bound to albumin in a 1:1 molar ratio for up to 2 hours. Assay Buffer #2 (Sodium chloride, potassium chloride, calcium chloride, magnesium sulfate, sodium bicarbonate,

HEPES, glucose and dipotassium phosphate) was used as the basolateral medium and contained 0.1 mM regular BSA, 0.1 mM fatty acid free BSA or no BSA. Samples (50 μl) were removed temporally from the basolateral medium and the radioactivity incorporated into the basolateral media determined. 50μl of basolateral media with 0.1 mM BSA was added back to the basolateral chamber to replenish the media. In some experiments, HBMEC were incubated basolaterally with 0.1mM [1-^{14}C]oleate bound to albumin in a 1:1 molar ratio and the appearance of [1-^{14}C]oleate in the apical medium containing 0.1 mM fatty acid free BSA was determined for up to 20 min. The apparent permeability coefficient for the various fatty acids in HBMEC monolayers was determined using the equation provided below:

Papp = dCr/dt (Vd/A * Cd)

where Cr is the concentration of DAR in the receiver compartment; t is time; Vd is the volume in the donor compartment; A is the surface area; and Cd is the concentration of DAR in the donor compartment at time 0.

The integrity of the HBMEC monolayers was determined spectrofluorometrically using 10μM fluorescein isothiocynate labeled dextran (FDX) 10,000 molecular weight (Invitrogen) as a paracellular diffusion marker. For these studies, HBMEC were incubated with 10 μM FDX and 1 μM, 10 μM, or 100 μM oleate for 2 hours. Basolateral medium was sampled temporally and fluorescence was determined using a Biotek Synergy HT plate reader. Fluorescence was detected at Ex (λ) 485 nm and Em (λ) 528 nm. Permeability was expressed as percent transfer of the fluorescent marker

across the cell monolayer.

In the fatty acid transport inhibitor studies HBMEC were incubated apically with 0.1 mM [1-^{14}C]oleate bound to albumin in a 1:1 molar ratio with or without 200 μM phloretin for 30 minutes. At various time points, 2, 5, 10, 20 and 30 minutes, 50 μl samples were removed from the basolateral medium and radioactivity determined. Radioactivity incorporated into phospholipids was also determined as described below.

2.5 Lipid extraction

Cells were harvested at each time point by washing twice with 2ml of ice-cold phosphate-buffered saline (PBS). The permeable inserts were cut and the cells were removed using a rubber policeman into 2ml of methanol:water (1:1 v/v). The mixture was vortexed, and a 25 μl aliquot was taken for protein analysis (Lowry *et al*, 1951) and a 10 μl aliquot taken for determination of total radioactivity. This process was repeated for both the basolateral media and apical media samples. Appropriate volumes of chloroform, 0.9% NaCl and methanol were added to the tubes in a 4:3:2 ratio to initiate phase separation. The tubes were vortexed and then centrifuged at 2,000 x g for 10 minutes (bench top centrifuge), the aqueous phase was removed and 2 ml theoretical upper phase (48 ml methanol, 47 ml 0.9% NaCl, 3 ml chloroform) was added to wash the organic phase. The tubes were vortexed and centrifuged as described above and the aqueous phase removed. The organic phase was dried under a stream of N_2 gas and

resuspended in 50 µl chloroform. A 20-40 µl aliquot of organic phase was placed onto a thin-layer plate along with phospholipid standards and phospholipids were separated by two-dimensional thin-layer chromatography. A 20-40 µl aliquot of organic phase was placed onto a thin-layer plate along with neutral lipid, cholesterol and cholesterol ester standards. The neutral lipids, cholesterol and cholesterol ester fractions were separated using a one-dimensional thin-layer chromatography. Radioactivity incorporated into phospholipids, neutral lipids, cholesterol and cholesterol esters were determined as described (Hatch and McClarty, 1996; Hatch *et al*, 2002). Radioactivity incorporated into prostaglandins were determined as described (Anders, 1969).

2.6 Capillary Depletion

An enriched capillary fraction was collected from mouse brain homogenates using the capillary depletion technique described by Triguero *et al*. (1990). Fresh mouse brains were isolated and the meninges removed. Brains were pooled together and the weight was recorded. Brain Buffer Solution, a physiological buffer solution consisting of 10mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 10 mM D-glucose and pH 7.4 was added at 5x the volume of the weight of the brain tissue. The brain tissue was then homogenized with a glass homogenizer in the brain buffer solution for 8 strokes. A 50 mg sample of the brain homogenate was removed as a representation of the whole brain. An equal volume of 26% dextran solution was added to the homogenate resulting in a final concentration of 13% dextran. This was then centrifuged at 5,400 x g for 15 minutes at 4^0C. Both the pellet and

supernatant were collected. The resulting pellet is referred to as the capillary enriched fraction; it consists of brain microvessel segments and red blood cells. The supernatant is referred to as the capillary depleted fraction; it consists of non-vascular central nervous system cells.

2.7 siRNA Transient Transfection

Transient transfection of HBMEC and HEK 293 cells was done using the Fast-Forward method for transfection of adherent cells as described in the HiPerFect reagent handbook (Qiagen). Briefly, cells were seeded at a density of 48,000 cells/cm^2 in T25 flasks in 4 ml of fresh medium 85,000 cells/cm^2 in 6-well inserts in 1.5 ml fresh medium or cells were seeded onto 100mm dishes at a density of $3.0x10^6$ cells/cm^2 in 10ml of fresh media. Cells were seeded 4-6 h prior to the addition of 5nM siRNA complexes. The FATP-1 siRNA target sequence was 5' – CCG GCT GGT GAA GGT CAA TGA. The FATP-3 siRNA target sequence was 5' – CAG GAG GTG AAC GTC TAT GGA. The FATP-4 siRNA target sequence was 5' – CCG CTT CGA TGG CTA CCT CAA. The FABP5 siRNA target sequence was 5' – AGG AGT TAA TTA AGA GAA TGA. The CD36 siRNA target sequence was 5' – CAG AAC CTA TTG ATG GAT TAA. Complexes of 5nM FATP-1, FATP-3, FATP-4, FABP5 or CD36 siRNA were formed by mixing siRNA with HiPerFect Reagent (Qiagen) and incubating for 10 min at room temperature. Cells were incubated with these complexes at 37^0C, 5.0% CO_2 for 48 h. Mock treated cells underwent the same transfection process but were incubated with only the HiPerFect reagent. Total RNA was isolated with trizol reagent

(GIBCO) to monitor FATP-1, FATP-3, FATP-4, FABP5 and CD36 gene silencing and measure the effectiveness of transfection. Mock & siRNA transfected HBMEC were incubated apically or basolaterally with 0.1mM [^{14}C]oleate bound to albumin in a 1:1 molar ratio for 20 min in the permeability studies and radioactivity incorporated into the basolateral or apical medium detected as described above. Mock & siRNA transfected HEK 293 cells were incubated with 0.1mM oleic acid bound to albumin (1:1 molar ratio) for 24 h.

2.8 Real-time RT-PCR Analysis

Total RNA from mock, FATP-1, FATP-3, FATP-4, FABP5 and CD36 siRNA transfected HBMEC and human grey and white matter brain tissue samples were isolated using Trizol reagent (GIBCO) according to the manufacturer's instructions. Measurement of gene expression by quantitative analysis was carried out using a Mastercycler ep *realplex* system (Eppendorf). Primers and hybridization probes were synthesized by Invitrogen (Ontario, Canada) and Qiagen (Massachusetts, USA). Quantitative real-time RT-PCR analysis of human FATP-1, FATP-2, FATP-3, FATP-4, FATP-5, FATP-6, FABP5, FABP7, CD36 and β-Actin gene expression was carried out using a Quantitect Probe RT-PCR SYBR Green kit (Qiagen). The sequences of the screening primers were; FATP-1, forward 5'- CCA CTT GGA TGT CAC CAC TG, reverse 5'- GTG GGA CCC TCC AGT AGA CA; FATP-2, forward 5' – ATG CGA GAA AAG TTG GTG CT, reverse 5' – TTT CAT CAC GGA CAG GTT CA; FATP-3,

forward 5’ – ATA CCT GGG AGC GTT TTG TG, reverse 5’ – CCG CTG TCC TGT GTA GTT GA; FATP-4, forward 5’ – GCT TCG ATG GCT ACC TCA AC, reverse 5’ – GTC CAT CAC CAG CAC CAT AC; FATP-5, forward 5’ – AGC TCC TGC GGT ACT TGT GT, reverse 5’ – AAG GTC TCC CAC ACA TCA GC; FATP-6, forward 5’ – GAA TTG GAG CAA TTG GGA GA, reverse 5’ – ACA CCA ACC CTG CTC ATT TC; CD36, forward 5’- AGA TGC AGC CTC ATT TCC AC, reverse 5’ – GCC TTG GAT GGA AGA ACA AA; FABP5, forward 5’ – ATG GCC AAG CCA GAT TGT AT, reverse 5’ – TGA ACC AAT GCA CCA TCT GT; FABP7, forward 5’ – CCA GCT GGG AGA AGA GTT TG, reverse 5’ – CTT TGC CAT CCC ATT TCT GT β-Actin, forward 5’- AGA AAA TCT GGC ACC ACA AC; reverse 5’- GGG GTG TTG AAG GTC TCA AA. PCR amplification of the housekeeping gene β-Actin was carried out for each sample as a control for sample loading and to allow normalization among samples. The PCR condition for each sample was 40 cycles of heating to 95^0C for 15 s, cooling to 60^0C for 30 s and 72^0C for 30 s. A melting curve was taken of each product to determine purity levels. To determine the relative gene expression ΔΔCt values were calculated by subtracting the ΔCt (sample) from the ΔCt (control). The β-Actin control was normalized to 1.0 and each target gene was then normalized and represented as the percentage of gene remaining.

Total RNA from mouse brain homogenates, capillary-enriched and capillary-depleted fractions were isolated using Trizol reagent (GIBCO) according to the manufacturer’s instructions. Measurement of gene expression by quantitative analysis was carried out

using a Mastercycler ep *realplex* system (Eppendorf). Primers and hybridization probes were synthesized by Invitrogen (Ontario, Canada) and Qiagen (Massachusetts, USA). Quantitative real-time RT-PCR analysis of murine FATP-1, FATP-2, FATP-3, FATP-4, FATP-5, FATP-6, FABP5, FABP7, CD36, Pgp, GFAP and β-Actin gene expression was carried out using a Quantitect Probe RT-PCR SYBR Green kit (Qiagen). The sequences of the screening primers were; FATP-1, forward 5'- ATT GTG GTG CAC AGC AGG TA, reverse 5' – GAT GTT CCC TGC AGA GTG GT; FATP-2, forward 5' – ATG CCG TGT CCG TCT TTT AC, reverse 5' – GAC CTG TGG TTC CCG AAG TA; FATP-3, forward 5' – AGG CTG CTC GAA TCA GTC AT, reverse 5' – AAC TTG GGT TTC AGC ACC AC; FATP-4, forward 5' – CAG CAA CTG TGA CCT GGA GA, reverse 5' – CCT TCC GCA ACT CTG TCT TC; FATP-5, forward 5' – TCG GAT CTG GGA ATT CTA CG, reverse 5' – CAG GAA TGC AAA AAC CCT GT; FATP-6, forward 5' – GGT CAC GGT GCT GGA TAA GT, reverse 5' – CGA GGA GTG GTT CAG GAG AG; FABP5, forward 5' – CAA AAC CGA GAG CAC AGT GA, reverse 5' – AAG GTG CAG ACC GTC TCA GT; FABP7, forward 5' – CCA GCT GGG AGA AGA GTT TG, reverse 5' – TTT CTT TGC CAT CCC ACT TC; CD36, forward 5' – CCT TAA AGG AAT CCC CGT GT, reverse 5' – TGC ATT TGC CAA TGT CTA GC; P-gp, forward 5' – GCG ACT CCG ATA CAT GGT TT, reverse 5' – ACC CTG TAG CCC CTT TCA CT; GFAP, forward 5' – CAC GAA CGA GTC CCT AGA GC, reverse 5' – GTA GGT GGC GAT CTC GAT GT. PCR amplification of the housekeeping gene β-Actin was carried out for each sample as a control for sample loading and to allow normalization among samples. The PCR

condition for each sample was 40 cycles of heating to 95^0C for 15 s, cooling to 60^0C for 30 s and 72^0C for 30 s. A melting curve was taken of each product to determine purity levels. To determine the relative gene expression ΔΔCt values were calculated by subtracting the ΔCt (sample) from the ΔCt (control). The β-Actin control was normalized to 1.0 and each target gene was then normalized and represented as the percentage of gene remaining.

RNA from oleate treated mock, FATP-1 knockdown and expressing HEK 293 cells was isolated using Trizol reagent (GIBCO) according to the manufacturer's instructions. Measurement of gene expression by quantitative analysis was carried out using a Mastercycler ep *realplex* system (Eppendorf). Primers and hybridization probes were synthesized by Invitrogen (Ontario, Canada) and Qiagen (Massachusetts, USA). Quantitative real-time RT-PCR analysis of human cytidine-5'-diphosphate-1,2-diacyl-*sn*-glycerol synthetase-2 (CDS-2), PGPS, CLS, FATP-1 and β-Actin gene expression was carried out using a Quantitect Probe RT-PCR SYBR Green kit (Qiagen). The sequences of the screening primers were; CDS-2, forward 5'- GTC AGC ATC CCT TTG TCG AT, reverse 5'- CCA AGC AAA CTG ATT CAG CA; PGPS, forward 5'- TCG GCC TCC AGC ACA TTA AG, reverse 5'- AGT CAC TCA GGT TTG CAC CG; CLS, forward 5'- CGA GAG ATG TAA TGT TGA TTG CTG, reverse 5'- CGA ACC GTG GTG TTG GAA GAG TT[FAM]G; FATP-1, forward 5'- CCA CTT GGA TGT CAC CAC TG, reverse 5'- GTG GGA CCC TCC AGT AGA CA; β-Actin, forward 5'- AGA AAA TCT GGC ACC ACA AC, reverse 5'- GGG GTG TTG AAG

GTC TCA AA. PCR amplification of the housekeeping gene β-Actin was carried out for each sample as a control for sample loading and to allow normalization among samples. The PCR condition for the each sample was 40 cycles of heating to 95^0C for 15 s, cooling to 60^0C for 30 s and 72^0C for 30 s. A melting curve was taken of each product to determine purity levels. To determine the relative gene expression between mock and FATP-1 expressing cells or mock and FATP-1 knockdown cells ΔΔCt values were calculated. ΔΔCt values were calculated by subtracting the ΔCt (sample) from the ΔCt (control). The β-Actin control was normalized to 1.0 and each target gene was then normalized following this equation. Results were represented as the mean of three RNA samples from each group.

2.9 Western Blot

HBMEC were transfected and incubated with FATP-1 or FATP-4 or CD36 or FABP5 siRNA for 48 h at 37^0C, 5.0% CO_2. Human brain tissue samples, 1996c38 and 1996c39, were donated by the Dr. Marc Del Bigio laboratory. Cells were harvested using cold PBS and pipetted into test tubes. The tubes were centrifuged at 500 x g for 10 min and the PBS removed. Addition of 0.5 ml homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCL, 0.145 M NaCl, pH 7.4) was followed by homogenization by 30 strokes of a tight fitting Dounce A homogenizer. Protein concentration was determined using Lowry's Method (Lowry *et al*, 1951). A 20 - 35 µg aliquot of the cellular fraction and 40 µg aliquot of the human brain tissue homogenate were subjected to SDS/PAGE (7.5% gels) with molecular-mass standards using a BioRad Mini-Protean® II Dual Slab

Cell electrophoresis unit. Proteins were transferred from the gel onto PVDF membranes by incubation for 100 min at 15 volts using a BioRad Trans-Blot SD Semi-Dry Transfer Cell. Expression of FATP-1, FATP-4, CD36 and FABP5 was examined by incubating the PVDF membrane with the anti-FATP-1 antibody (1:500 dilution), anti-FATP-4 antibody (1:333 dilution), anti-CD36 antibody (1:500 dilution) and anti-FABP5 antibody (1:200 dilution) dissolved in Tris-buffered saline containing 0.1% Tween 20 overnight at 4 °C with shaking. Subsequently, the membrane was washed and incubated with a horseradish peroxidase labeled anti-mouse (against FATP-1) and anti-rabbit (against FATP-4, CD36 and FABP5) secondary antibody (1:1500) for 1 h at room temperature (23 °C). Expression of β-actin was examined by incubating the PVDF membrane with the antibody as previously described (Webster *et al*, 2005). Protein bands in the membrane were visualized by enhanced chemiluminescence. The relative intensities of the bands were analyzed by scanning the film, and subsequently determined by Scion Image software.

2.10 Preparation of mitochondrial fractions and enzyme assays

All isolation procedures were performed at 4°C. Cells were incubated with 0.1mM oleic acid bound to albumin (1:1 molar ratio) for 24 h. Cells were harvested using PBS and pipetted into test tubes. The tubes were centrifuged at 500 x g for 10 min and the PBS removed. Addition of 1 ml homogenizing buffer (0.25 M sucrose, 10 mM Tris-HCL, 0.145 M NaCl, pH 7.4) was followed by homogenization by 50 strokes of a tight fitting

Dounce A homogenizer. The homogenate was centrifuged at 1,000 x g for 10 min and the resulting supernatant centrifuged at 12,000 x g for 15 min. The resulting pellet was resuspended in 0.5 ml of homogenizing buffer by a tight fitting Dounce A homogenizer and used as the source of mitochondrial fraction for assay of mitochondrial enzyme activities. CDS, PGPS and CLS activities were determined as previously described (Hatch and McClarty, 1996).

2.11 Statistics

All data were expressed as mean $\pm$ S.D. The comparisons between the experimental and the control groups were evaluated by Student's t-test. Comparisons between more than two groups were evaluated by one-way ANOVA followed by the Tukey test. Values showing $p < 0.05$ were considered statistically significant unless otherwise indicated in the text.

2.12 Other determinations

Isolation and determination of long-chain acyl-CoA from mitochondrial fractions of HEK 293 mock and C8 cells expressing FATP-1 were previously described (Tardi *et al*, 1992). Acyl-CoA content in the samples were determined using gas chromatography and data analyzed by a Shimadzu Chromatopac CR501. Protein was measured by the method of Lowry (Lowry *et al*, 1951). In some experiments, equivalent protein

amounts of mock and FATP-1 expressing cells were separated by two-dimensional isoelectric focussing/sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained as described (Wall and Bailey, 1985).

3 RESULTS

To determine the permeability of the HBMEC monolayer across the Transwell® inserts, flux of FDX 10000 across blank and HBMEC inserts was determined. FDX flux was only 1.5 % at 30 min when HBMEC are present as a confluent monolayer on the inserts compared to 15.6 % when the insert is blank (**Figure 4**). After 1 h the FDX flux was only 6.1 % across HBMEC monolayers compared to 17.6 % across blank inserts (**Figure 4**). Thus HBMEC provide a tight monolayer across the Transwell® inserts with negligible FDX flux up to 30 min.

To examine the effects of oleate on HBMEC monolayer integrity, permeability was examined in the absence or presence of various concentrations of oleate (bound to albumin in a 1:1 molar ratio) using FDX 10000 as a permeability marker. Under control conditions, the average percentage flux of FDX was negligible at 30 min and was 8.1% after 1 h and 12.8% after 2 h (**Figure 5**). In addition, there were no differences in the flux of FDX between control monolayers and HBMEC monolayers treated with the various oleate concentrations. Thus, the HBMEC monolayer was intact and the presence of $\leq$ 0.1 mM oleate in the apical medium did not have an effect on the permeability of the HBMEC monolayers.

To examine fatty acid passage across HBMEC, confluent monolayers were incubated apically with 0.1mM [1-^{14}C]oleate (bound to albumin 1:1) for 2 h and radioactivity incorporated into the basolateral medium determined. The basolateral medium

contained either no BSA or 0.1 mM fatty acid free BSA. In the absence of BSA, incorporation of radioactivity into the basolateral medium was negligible at 30 min and reached only 4.5% of total radioactivity added to the apical compartment after 2 h of incubation (**Figure 6**). In contrast, in the presence of BSA, incorporation of radioactivity into the basolateral medium was significantly elevated with time, 10-fold ($p<0.05$) by 30 min of incubation compared to medium devoid of BSA. The incorporation of radioactivity into the basolateral medium was significantly higher at 2 h compared to that at 30 min in the presence of BSA. The BSA did not need to be devoid of fatty acid as there was no difference in radioactivity incorporated into the basolateral medium when regular BSA was used in place of fatty acid free BSA (data not shown). In addition, >99% of total radioactivity in the basolateral medium was [1-^{14}C]oleate. Thus, oleate transport across the HBMEC requires a protein acceptor in the basolateral medium.

The metabolic fate of oleate was also determined in HBMEC harvested at the conclusion of the permeability studies. In these studies, [1-^{14}C]oleate was incorporated into all phospholipids of HBMEC with most radioactivity incorporated into phosphatidylcholine (PtdCho) (**Table 1**). The basolateral medium was then analyzed for lipid content. Although PC was highly synthesized from [1-^{14}C]oleate within HBMEC, a near negligible amount, 0.3% of PtdCho cellular radioactivity was observed in the basolateral medium. PtdCho is the major membrane phospholipid of HBMEC and its near absence in the basolateral medium indicated that cell membrane lipid components

were not being sloughed off the insert and was further evidence that the monolayer remained intact. In another set of experiments the cholesterol and cholesteryl ester fractions were determined following 0.1mM [1-^{14}C]oleate (bound 1:1 to albumin) exposure. In these studies, the cholesterol fraction comprised only 2.1 % of the total lipids formed and cholesteryl ester fraction comprised 0.95 % of the total lipids formed.

To directly determine the net flow of fatty acid across the HBMEC monolayer, permeability coefficients were determined for [1-^{14}C]oleate incorporation into the basolateral medium and apical medium. HBMEC were incubated with 0.1 mM [1-^{14}C]oleate bound 1:1 to albumin in either the apical or basolateral media for up to 1 h. Radioactivity incorporated into either the basolateral and apical media was determined temporally and the permeability coefficients were calculated. The permeability coefficient from apical to basolateral media (A-B) was 2.6 x 10^{-5} cm/s . The permeability coefficient from basolateral to apical media (B-A) was almost half that of the A-B coefficient, 1.6 x 10^{-5} cm/s (**Figure 7**). The B-A/A-B ratio was 0.64, indicating that there is a net influx of oleate from the apical side to the basolateral side across the HBMEC monolayer.

Transport of fatty acids with various chain lengths, and degree of unsaturation were examined. HBMEC were incubated with 0.1mM of various radiolabeled fatty acids bound 1:1 to albumin for up to 30 min and radioactivity incorporation into the basolateral medium determined. Butyric acid (C4:0), a short chain fatty acid, readily

crossed the HBMEC monolayer into the basolateral medium (**Figure 8**). Over 52 % of the total [1-^{14}C]butyric acid added to the apical medium accumulated in the basolateral medium after 30 min incubation. [1-^{14}C]myristic acid (C14:0), a medium-chain saturated fatty acid, followed a similar level of accumulation as the unsaturated fatty acids. 41 % of the total [1-^{14}C]myristic acid added to the apical medium accumulates in the basolateral medium after 30 min. Palmitic acid (C16:0), a long-chain saturated fatty acid, accumulated in the basolateral medium at a much slower rate. Only 21 % of the total [1-^{3}H]palmitic acid added to the apical medium accumulated in the basolateral medium. Lignoceric acid (C24:0), a very-long-chain saturated fatty acid did not appear to cross the HBMEC monolayer, with only 5 % of the total [1-^{14}C]lignoceric acid accumulating in the basolateral medium.

The unsaturated fatty acids, [1-^{14}C]oleic acid (C:18:1), [1-^{14}C]linoleic acid (C18:2) and [1-^{3}H]arachidonic acid (C22:4) readily accumulated in the basolateral medium at similar rates, with 39 %, 36 % and 37 % of their respective total radioactivity added to the apical medium observed in the basolateral medium after 30 min (**Figure 8**). Thus, it appeared that chain length of the saturated fatty acids as well as the degree of saturation predicts fatty acid apical to basolateral permeability across HBMEC monlayers.

The mechanism of oleate transport across HBMEC was then examined. HBMEC were incubated with 0.1 mM [1-^{14}C]oleate and 0-0.1 mM unlabeled oleate bound 1:1 to albumin for 2 h and radioactivity incorporated into the cells determined. As the

unlableled oleate concentrations were increased the radioactivity incorporation into the cells decreased, suggesting that oleate uptake into HBMEC was, at least in part, a carrier-mediated process (**Figure 9)**. Uptake of oleate was saturable with Km = 124.2 $\pm$ 18.95 μM and Vmax = 427.1 $\pm$ 36.97 pmol/min/mg protein (**Figure 9 inset**).

To further explore the carrier-mediated process for oleate transport in HBMEC, cells were exposed to the non-specific fatty acid uptake inhibitor, phloretin. Confluent HBMEC monolayers were incubated apically with 0.1 mM [1-^{14}C]oleate (bound 1:1 to albumin) plus or minus 200 μM phloretin for 30 min and radioactivity incorporated into the basolateral medium was determined. In the presence of phloretin, incorporation of oleate into the basolateral medium was reduced at all times examined and was significantly reduced by 70.5±?% ($p<0.05$) and by 78.5±?% ($p<0.05$) by 25 min and 30 min of incubation, respectively, compared to control (**Figure 10**). Thus, transport of oleate across HBMEC was, in part, a carrier mediated process which may involve fatty acid transport proteins.

Next, we examined the fatty acid transporter expression profile of HBMEC (**Table 2**). FATP-1, -3 and -4 were expressed in HBMEC. FATP-1 and FATP-4 were expressed at the highest level followed by FATP-3. Expression of FATP-5 was low and barely detectable. FATP-2 and FATP-6 expression levels were not detectable in HBMEC. In addition, FAT/CD36 and FABP5 were expressed in HBMEC. Expression of FABP7 was low and barely detectable. Fatty acid transporter expression profile was also

determined in mouse brain capillary-enriched and capillary-depleted fractions (**Table 2**). The capillary-enriched fraction was confirmed by the high expression of P-gp and low expression of GFAP. The capillary-depleted fraction was confirmed by the expression of GFAP. The capillary-enriched fraction had a fatty acid transporter expression profile similar to that of HBMEC. FATP-1 and FATP-4 were the highest expressed followed by FABP5. In general, fatty acid transporters were more highly expressed in the capillary-enriched fraction compared to the capillary-depleted fraction. In contrast, FABP7 was highly expressed in the capillary-depleted fraction and was expressed to a much lower extent in the capillary-enriched fraction.

To determine if fatty acid transporters in human brain tissue had a similar expression profile to that of HBMEC and mouse capillaries fatty acid transporters expression profile in human white and grey matter brain tissue samples was determined (**Table 3**). For these experiments, grey matter represents the capillary-enriched portion of the brain as it mainly consists of the capillaries, neuronal cell bodies and glial cells. Whereas for the purposes of these experiments the white matter represents the capillary-depleted portion of the brain as it consists of mainly myelinated axons. FATP-1, FATP-4 and FABP5 were highly expressed in the grey matter and the remaining fatty acid transport proteins studied were expressed in low amounts. Fatty acid transporters were more highly expressed in the grey matter compared to the white matter.

FATP-1 protein expression was then determined in human brain tissue homogenates

(**Figure 11**). We focused on FATP-1 since this fatty acid transporter was most highly expressed in HBMEC, human brain grey matter and in the capillary-enriched fraction of mouse brain. FATP-1 protein expression was observed in two different human brain tissue samples, 1996c38 and 1996c39.

Since phloretin is a non-specific inhibitor of various cellular transport processes, the role of inhibition of two common but specific fatty acid transport proteins, FATP-1 and CD36, on oleate transport into the basolateral medium was examined. The HBMEC were transiently transfected with FATP-1 or CD36 siRNA for 48 h, and expression of FATP-1 or CD36 determined using real time-PCR. Both FATP-1 and CD36 mRNA expression were significantly reduced 58% ($p<0.05$) and 52% ($p<0.05$), respectively, in HBMEC's transiently transfected with FATP-1 or CD36 siRNA compared to mock transfected cells (**Figure 12A**). Western blot analysis indicated a significant knockdown at the protein level as well (**Figure 12B**). FATP-1 protein was significantly reduced 41% ($p<0.05$) and CD36 protein was significantly reduced 29% ($p<0.05$) in HBMEC transiently transfected with FATP-1 or CD36 siRNA. To determine what effect FATP-1 or CD36 knockdown had on HBMEC permeability, monolyer integrity was assessed with FDX 10000. The passage of FDX 10000 across HBMEC monolayers following FATP-1 or CD36 knockdown was similar to that observed in the mock transfected controls (**Figure 12C**). Thus, FATP-1 or CD36 knockdown did not alter the general integrity of HBMEC monolayers. In contrast, significant reductions in 0.1 mM [1-^{14}C]oleate permeability was observed in the FATP-1 and CD36 siRNA transfected

HBMEC monolayers. Radioactivity in the basolateral medium was significantly reduced 34% ($p<0.05$) and 78% ($p<0.05$) by 10 min and 20 min of incubation, respectively, in FATP-1 siRNA transfected cells compared to mock transfected controls (**Figure 12D**). In addition, radioactivity in the basolateral medium was significantly reduced 68% ($p<0.05$) and 83% ($p<0.01$) by 10 min and 20 min of incubation, respectively, in CD36 siRNA transfected cells compared to mock transfected controls. Thus, transport of oleate from the apical to the basolateral medium across HBMEC is mediated, in part, by FATP-1 and CD36 fatty acid transport proteins.

Transient siRNA knockdown of other fatty acids transporters expressed in HBMEC were then examined. HBMEC were transiently transfected with FATP-3, FATP-4 or FABP5 siRNA for 48 h, and expression of FATP-3, FATP-4 or FABP5 determined using real time-PCR. FATP-3 mRNA expression was significantly reduced 50% ($p<0.05$), FATP-4 mRNA expression was also significantly reduced 51% ($p<0.05$) in HBMEC's transiently transfected with FATP-3 or FATP-4 siRNA compared to mock transfected cells (**Figure 13A**). Similar knockdown is observed in HBMEC transiently transfected with FABP5 siRNA. FABP5 mRNA expression was significantly reduced 47% in HBMEC transiently transfected with FABP5 compared to mock transfected cells (**Figure 13A**). Western blot analysis confirmed knock down (**Figure 13B**). FATP-4 protein expression was (39% $p<0.05$) significantly reduced in HBMEC transiently transfected with FATP-4 siRNA and FABP5 protein expression was (32% $p<0.05$) significantly reduced in HBMEC transiently transfected with FABP5 siRNA. To

determine what effect FATP-3, FATP-4 or FABP5 knockdown had on HBMEC permeability, monolayer integrity was assessed with FDX 10000. The passage of FDX 10000 across HBMEC monolayers following FATP-3, FATP-4 or FABP5 knockdown was similar to that observed in the mock transfected controls (**Figure 13C**).

The effect of knocking down expression of these transport proteins on the permeability of various fatty acids across HBMEC were determined. HBMEC were transiently transfected with FATP-1 or FATP-3 or FATP-4 or CD36 or FABP5 siRNA for 48 h and then incubated up to 10 min with 0.1 mM radiolabeled fatty acid bound to albumin in a 1:1 molar ratio and radioactivity incorporated into the basolateral medium determined. A summary of this knock down is presented in **Table 4**. CD36 appears to play a major role in fatty acid transport across the HBMEC as transport of a number of different fatty acids were reduced (**Figure 14A-F**). FATP-1 appeared to be more specific for long-chain fatty acids, such as oleic acid and palmitic acid as transport of these fatty acids were reduced by FATP-1 knock down (**Figure 12D** and **Figure 14C**). However, FATP-1 knockdown had no effect on [1-^{14}C]linoleic acid transport indicating that degree of unsaturation may be important for fatty acid transport utilizing FATP-1 in HBMEC (**Figure 14F**). In contrast, [1-^{14}C]linoleic acid transport was significantly reduced when FATP-4 and FABP5 were transiently knocked down with siRNA compared to the mock transfected control cells. FABP5 appeared to be more specific for long-chain fatty acids as well regardless of the degree of unsaturation unlike FATP-1. Transport of palmitic acid, oleic acid and linoleic acid were reduced by FABP5 knock down (**Figure**

14C,E,F). Transport and accumulation of [1-^{14}C]lignoceric acid across HBMEC was very low, however, CD36 knockdown appeared to significantly reduce its transport across HBMEC transiently transfected with CD36 siRNA compared to mock transfected HBMEC (**Figure 14D**).

[1-^{3}H]arachidonic acid transport across HBMEC did not appear to be affected by the knock down of FATP-1 or CD36 by siRNA (**Figure 15A**). However, the FDX 10000 flux was significantly higher when cells were treated with arachidonic acid (**Figure 15B)**. Arachidonic acid is the precursor to prostaglandins and these prostaglandins are known to increase permeability of BBB monolayers (Davidson *et al*, 2001; Jaworowicz *et al*, 1998). Prostaglandin synthesis was determined in HBMEC treated with [1-^{3}H]arachidonic acid bound to albumin 1:1 for up to 10 min in the basolateral medium. There was significant prostaglandin E_2 (PGE_2) production in cells incubated with [1-^{3}H]arachidonic acid (223.12 $\pm$ 33.74 dpm/mg protein) . Thus, there appeared to be some arachidonic acid converted to PGE_2 in our incubations and this may have accounted for why arachidonic acid rapidly accumulated in the basolateral medium.

We next examined if these fatty acid transporters altered oleate movement from the basolateral to the apical side of HBMEC's. The HBMEC were transiently transfected with FATP-1 or CD36 siRNA for 48 h and then incubated for up to 20 min with 0.1 mM [1-^{14}C]oleate (bound to albumin in a 1:1 ratio) in the basolateral medium and radioactivity incorporated into the apical medium determined. Radioactivity in the

apical medium was significantly reduced 52% ($p<0.05$) and 54% ($p<0.05$) by 10 min and 20 min of incubation, respectively, in FATP-1 siRNA transfected cells compared to mock transfected controls (**Figure 16**). In addition, radioactivity observed in the apical medium was significantly reduced 25% ($p<0.05$) and 42% ($p<0.05$) by 10 min and 20 min of incubation, respectively, in CD36 siRNA transfected cells compared to mock transfected controls. Thus, transport of oleate from the basolateral to the apical medium across HBMEC is also mediated, in part, by FATP-1 and CD36 fatty acid transport proteins. In addition, these transport proteins may be localized to both apical and basolateral membranes of HBMEC. Interestingly, in the mock transfected HBMEC the total radioactivity in the apical medium was several-fold lower than in the previous basolateral experiments after 20 min (**Figures 12 and 16**). Thus, there appeared to be more fatty acid protein-mediated transport occurring at the apical membrane.

Since fatty acid import into cells plays a key role in fatty acid oxidation and CL is required for oxidative phosphorylation, we examined whether altering the level of FATP-1 regulated *de novo* CL biosynthesis. HEK 293 cells were transfected with FATP-1 siRNA for 48 h and FATP-1 mRNA expression was determined by real time-PCR. FATP-1 mRNA expression was significantly knocked down (**Figure 17**). Mock and FATP-1 siRNA transfected cells were then incubated with 0.1mM oleate (bound to albumin 1:1) for 24h and then with 0.1 mM [1,3-^{3}H]glycerol for 24 h and the radioactivity incorporated into phospholipids determined. Incorporation of [1,3-^{3}H]glycerol into CL was increased 2-fold ($p<0.05$) in FATP-1 siRNA transfected cells

compared to mock transfected controls (**Table 5**). In addition, [1,3-^{3}H]glycerol incorporated into other glycerophospholipids was also increased approximately 2-fold ($p<0.05$). Total uptake of [1,3-^{3}H]glycerol was increased 2-fold ($p<0.05$) in FATP-1 siRNA transfected cells compared to mock transfected controls. These data indicated that knock down of FATP-1 in HEK 293 cells stimulated CL and phospholipid *de novo* synthesis from glycerol at the level of glycerol uptake.

Mock and FATP-1 siRNA transfected cells were then incubated with 0.1 mM oleate (bound to albumin 1:1) for 24h and then with 0.1 mM glycerol for 24 h and the pool size of CL and the major phospholipids, phosphatidylethanolamine (PE) and phosphatidylchoine (PC) determined. The pool size of CL was increased 2.3-fold ($p<0.05$) in FATP-1 siRNA transfected cells compared to controls (**Figure 18A**). However, the overall phospholipid content in the cells did not change as no alterations in the pool size of the major phospholipids PE and PC were observed. Thus, *de novo* CL synthesis was increased in FATP-1 siRNA transfected HEK 293 cells incubated with oleate.

The reason for the increase in CL was examined. Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate (bound to albumin 1:1) for 24 h and then with 0.1 mM glycerol for 24 h mitochondrial fractions prepared and the activities of the enzymes of the CDP-DG pathway determined. CDS, PGPS and CLS activities were increased 23% ($p<0.05$), 21% $p<0.05$) and 71% ($p<0.05$), respectively, in FATP-1

siRNA transfected cells compared to controls (**Table 6A**). Thus, the increase in CL content in FATP-1 siRNA transfected cells was due to an increase in the activities of the enzymes of the CDP-DG pathway of CL synthesis.

The reason for the increase in enzyme activities of CDS, PGPS and CLS in oleate-treated in FATP-1 siRNA transfected cells was examined. Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate (bound to albumin 1:1) for 24h and then with 0.1 mM glycerol for 24 h and total mRNA prepared and expression of CDS-2, PGPS and CLS mRNA examined. CDS-2, PGPS and CLS mRNA expression were all elevated in the FATP-1 siRNA transfected cells to the mock transfected cells (**Figure 19A-19C**). Thus, the content of CL and increased [1,3-^{3}H]glycerol incorporation into CL in oleate-treated FATP-1 siRNA transfected cells was due to an increase in activity and mRNA expression of enzymes of the CDP-DG pathway of CL synthesis.

Since FATP-1 knockdown in HEK 293 cells resulted in an increase in CL synthesis we examined if expression of FATP-1 in HEK 293 cells had the corollary effect on CL synthesis. First, we examined the protein expression profile of both mock and FATP-1 expressing cells (C8 cells) by two-dimensional isoelectric focusing/sodium dodecyl sulphate polyacrylamide gel electrophoresis (**Figure 20**). Next, mock or C8 cells expressing FATP-1 were incubated with 0.1 mM oleate (bound to albumin 1:1) for 24 h and then with 0.1 mM [1,3-^{3}H]glycerol for 24 h and radioactivity incorporation into CL determined. Incorporation of [1,3-^{3}H]glycerol into CL was reduced 65% ($p<0.05$) in C8

cells compared to control (**Table 7**). In addition, [1,3-^{3}H]glycerol incorporated into PG and PC was decreased. In contrast, total uptake of [1,3-^{3}H]glycerol was unaltered between C8 cells and controls. These data indicated that expression of FATP-1 in HEK 293 cells inhibited CL synthesis from glycerol.

The pool size of CL and the major phospholipids in FATP-1 expressing cells were examined. The pool size of CL and the other major phospholipids (PE and PC) were unaltered between vector and C8 cells treated with oleate (**Figure 18B**). Thus, *de novo* CL biosynthesis from [1,3-^{3}H]glycerol was decreased in C8 cells expressing FATP-1 incubated with oleate but this did not affect the CL pool size. The reason for the decrease in [1,3-^{3}H]glycerol incorporation into CL in C8 cells was examined. Mock and C8 cells were incubated with 0.1 mM oleate for 24 h and mitochondrial fraction isolated and CL biosynthetic enzyme activities determined. CDS activity was reduced 38% ($p<0.05$) in C8 cells compared to controls (**Table 6B**). PGPS and CLS activities were unaltered between control and C8 cells. The reason for the decrease in CDS activity in C8 cells was examined. Mock and C8 cells were incubated with 0.1 mM oleate for 24 h and total RNA isolated and CDS-2 mRNA expression determined. CDS-2 mRNA expression was decreased in C8 cells compared to controls (**Figure 21**). Thus, the decreased CL synthesis in oleate-treated C8 cells was due to a reduction in CDS enzyme activity and CDS-2 mRNA expression.

FATP-1 exhibits acyl-Coenzyme A synthetase activity (Hall *et al*, 2003) and expression

of FATP-1 in C8 cells was shown to elevate intracellular fatty acids levels (Hatch *et al*, 2002). A previous study had indicated that CDS enzyme activity was inhibited by oleic acid in isolated lymph node lymphocytes (Sribney and Hegadorn, 1982). Mitochondrial fractions from HEK 293 cells were prepared and CDS enzyme activity determined in the absence or presence of various concentrations of oleoyl-Coenzyme A. CDS activity was reduced in the presence of 1 μM or greater concentrations of oleoyl-Coenzyme A (**Figure 22A**). This data indicated that the reduction in the CDS enzyme activity seen in C8 cells expressing FATP-1 might also be in part due to increases in fatty acyl-Coenzyme A in these cells (**Figure 22B**).

4 DISCUSSION

Transport of fatty acids into the brain and other tissues is still highly debated and controversial. Hamilton (1998) suggested that fatty acids do not require fatty acid transport proteins in order to pass across cellular membranes, whereas, Abumrad *et al* (1998) provided evidence of facilitated transport of fatty acids by proteins. The objective of this study was to examine the mechanism of transport of fatty acids across brain microvessel endothelial cells using a simulated blood brain barrier model.

HBMEC provide an intact monolayer that severely restricts the passage of certain substances. The passage of FDX 10000 was dramatically reduced in the presence of HBMEC on the Transwell® inserts. As previously stated, the integrity of the monolayer is crucial for the subsequent studies on fatty acid transport, HBMEC acts to strengthen the restrictiveness of the insert in our model. The overall flux of fatty acids across the HBMEC monolayer was determined by calculating the permeability coefficients of radiolabeled oleate incubated in the apical and the basolateral media. The permeability coefficient of [1-^{14}C]oleate from the apical to basolateral media was nearly double that of the permeability coefficient of [1-^{14}C]oleate from the basolateral to apical media. The ratio of the permeability coefficients was less than 1, indicating that there is a net influx of oleate into the basolateral media. This suggests that there is a net influx of oleate into the brain from the blood and that the transport of fatty acids into the brain is of higher importance than the export of fatty acids into the blood.

The addition of albumin to the system demonstrated that a protein acceptor, in this case bovine serum albumin (BSA), was required in the basolateral medium in order for oleate to be efficiently transported across HBMEC. Transport of [1-^{14}C]oleate was increased when BSA was present in the basolateral medium. Albumin comprises up to 50-70% of the protein in the normal cerebral spinal fluid (Di Terlizzi and Platt, 2006). In addition, the albumin concentration in human cerebral spinal fluid ranges from 55-1960 mg/l (Marshall and Williams, 2000). Thus, albumin could act as a sink for fatty acid transport across the BBB. Moreover, it is possible that membrane proteins in astrocytes and pericytes in close proximity to HBMEC could also act as potential acceptors *in vivo*.

The average percentage flux of FDX after 2 h for each concentration of oleate used was identical to that of control indicating that the HBMEC monolayer remained intact. The metabolic fate of oleate in HBMEC and the metabolites released into the basolateral medium were also indicative of an intact and unperturbed cell monolayer. PC is a major membrane phospholipid and incorporates the majority of oleate radioactivity in cells (Groener *et al*, 1996). Incorporation of [1-^{14}C]oleate into PC was high in HBMEC's lysates but minimal PC radioactivity was observed in the basolateral media. The near absence of PC in the basolateral medium indicated that cell membrane lipid components were not being sloughed off and would account for the radioactivity in the basolateral medium. In addition, [1-^{14}C]oleate accounted for >99% of the radioactivity in the basolateral medium. Thus, transport of fatty acids across the BBB monolayer appears to

be independent of its metabolism, consistent with studies done by Kamp *et al*, 2003 and Guo *et al*, 2006.. A previous study showed that oleate rapidly infused into the carotid artery of rats at a rate that would bypass albumin binding reversibly disrupted the BBB (Sztriha and Betz, 1991). In the current study, we incubated cells with oleate bound to albumin in a 1:1 molar ratio, thus eliminating the disruptive effect of free oleate on HBMEC.

The saturation of fatty acids and the fatty acid chain length alter the transport and passage of fatty acids across the HBMEC monolayer. Short to medium chain length saturated fatty acids, such as butyric acid and myristic acid, were able to enter into the basolateral medium in a higher amount than long chain and very-long chain saturated fatty acids, such as palmitic acid and lignoceric acid. Perdeuterated palmitic acid and stearic acid (C18:0) fed to rat pups failed to accumulate in the brain but were found intact in liver, lungs and kidneys (Edmond *et al*, 1998). Unsaturated fatty acids, such as oleate, linoleic acid and arachidonic acid, were able to cross the HBMEC monolayer and accumulate in the basolateral medium in a higher amount than the saturated fatty acids of similar chain lengths such as palmitic acid. However, arachidonic acid appeared to disrupt the HBMEC monolayer and increase the permeability to the paracellular marker FDX. Previous studies have demonstrated an increase in permeability of the cerebromicrovascular endothelium through the addition of free arachidonic acid (Villacara *et al*, 1989). In the current study it is believed that arachidonic acid was quickly metabolized to prostaglandins by HBMEC. Prostaglandins

have been linked to disrupting the integrity of the HBMEC monolayer (Davidson *et al*, 2001; Jaworowicz *et al*, 1998). Radioactive PGE_2 production was observed in the basolateral medium of HBMEC treated with [1-^{3}H]arachidonic acid indicating that an elevation in prostaglandin production may have been responsible for the increased permeability in arachidonic acid treated HBMEC monolayer.

As the concentration of unlabeled oleate was increased in the apical medium the [1-^{14}C]oleate incorporated into HBMEC decreased. These findings indicated that oleate was transported across HBMEC via a carrier/transporter-mediated process. Further evidence in support of this was the observation that phloretin, a non-specific fatty acid uptake inhibitor, reduced [1-^{14}C]oleate incorporation into the basolateral medium. Together, these data indicated that oleate transport across HBMEC is likely a transcellular process. It is recognized that phloretin is non-specific for fatty acid uptake inhibition and has many other diverse cellular effects including inhibition of monosaccharide and anion membrane transport, acceleration of lipophilic transport and alteration of ion transport (Verkman and Soloman, 1982).

The expression profile of the fatty acid transporter proteins in HBMEC, human brain tissue homogenates and mouse capillary-enriched fractions were determined. FATP-1 and FATP-4 mRNA were both highly expressed in the HBMEC as well as in the capillary-enriched fraction of the mouse brain tissue. Northern blot analysis of murine *FATP* gene family produced similar results, in which FATP-4 and FATP-1 were the

only FATPs found in the brain (Hirsch *et al*, 1998). FATP-4 has also been reported as the primary FATP expressed in the brain (Fitscher *et al*, 1998). In the present study, similar protein expression levels of FATP-1 were found in the whole human brain homogenates samples, suggesting that FATP-1 is not only highly expressed in the vascular portion of the brain but also in the non-vascular portion of the brain. This is supported through Northern blot analysis of FATP-1 in the mouse brain (Schaffer and Lodish, 1994). In addition, FATP-1 and FATP-4 mRNA were both highly expressed in the vascular grey matter of human brain tissue samples compared to white matter, suggesting their importance in the microvasculature but also in neuronal cell bodies and glial cells. FATP-3 is found in a wide variety of tissues; lungs, liver and testis (Hirsch *et al*, 1998). In this study FATP-3 was also found to be moderately expressed in HBMEC. FATP-3 expression in the mature brain had not been previously reported, however, previous studies have used whole brain samples and have not determined FATP expression in the endothelial microvasculature of the brain. FATP-5 expression in HBMEC was negligible as it is found exclusively in liver (Hirsch *et al*, 1998). Both FATP-2 and FATP-6 mRNA expression were not detectable in HBMEC. FATP-2 is exclusively expressed in the liver and the kidney cortex (Hirsch *et al*, 1998) and FATP-6 is the primary FATP found in the heart (Gimeno *et al*, 2003; Watkins *et al*, 1999).

CD36 was moderately expressed in HBMEC and its expression was slightly elevated in human grey matter compared to white matter. CD36 expression in endothelial cells of the microvasculature has been well established (Husemann *et al*, 2002) and its

importance in the brain has been reviewed in Abumrad *et al*, 2005. FABP5 was more highly expressed than FABP7 in HBMEC and the capillary-enriched mouse brain samples. Similarly, FABP5 is highly expressed in the endothelial cells of the microvasculature (Masouyé *et al*, 1997). FABP7 potentially has a more important role in the neurons and glial cells as it was more highly expressed in the capillary-depleted mouse brain fraction compared to HBMEC and capillary-enriched fraction. As stated earlier, both FABP5 and FABP7 are found in glial cells in the central nervous system of both pre- and perinatal brain (Owada *et al*, 1996; Veerkamp and Zimmerman, 2001).

Given the absence of specific fatty acid transport inhibitors, siRNA knockdown studies were performed. The targets of the knock-down were FATP-1, FATP-3, FATP-4, CD36 and FABP5. FATP-1 and CD36 are both highly expressed in the microvascular endothelium of the BBB (Schaffer and Lodish, 1994; Doege and Stahl, 2005; Husmann *et al*, 2002). The high expression levels of both FATP-1 and CD36 suggest that they are very important for both fatty acid transport as well as fatty acid metabolism in the BBB.

FATP-1, FATP-4 and FABP5 appear to have more selective roles in fatty acid transport across HBMEC. FATP-1, FATP-4 and FABP5 knockdown significantly reduced the transport of long-chain fatty acids, such as oleic acid, palmitic acid and linoleic acid. Knockdown of CD36 reduced the transport of all fatty acids examined. CD36 has a high affinity for long-chain fatty acid transport (Abumrad *et al*, 1993). Overexpression of FAT/CD36 resulted in an increased rate of fatty acid uptake (Ibrahimi *et al*, 1999)

whereas FAT/CD36 null mice exhibited a reduced fatty acid uptake (Coburn *et al*, 2000; Febbraio *et al*, 1999). However, knockdown of CD36 in HBMEC resulted in reduced transport of short-chain, medium-chain and long-chain fatty acids (butyric acid, myristic acid and oleic acid and linoleic acid) indictating that CD36 appeared to play a more general role in fatty acid transport across the HBMEC monolayer. FATP-3 knockdown does not appear to have a functional role in the transport of the fatty acids used in this study across HBMEC. FATP-3 knockdown studies in mouse Leydig cells (MA-10) produced similar results, fatty acid uptake was unaffected in these cells (Pei *et al*, 2004).

Finally, siRNA knockdown of the specific fatty acid transporters FATP-1 and CD36 reduced [1-^{14}C]oleate transport into the apical medium indicating that HBMEC may be capable of exporting fatty acids as well as importing fatty acids and that these transport proteins may be localized to both the apical side and basolateral sides of the permeability barrier. Although, based on our data there appears to be more apical to basolateral transport as opposed to basolateral to apical transport of oleate. We have demonstrated the actual amount of radiolabeled fatty acid found in the apical medium was one-fifth of the amount of radiolabeled fatty acid found in the basolateral medium. This observation indicates that these transport systems may be more important for importing fatty acids into the brain than exporting fatty acids from the brain into the blood.

There are limitations with the use of a static Transwell® model compared to the BBB *in vivo* setting. For example, the trans-endothelial electrical resistance (TEER) is lower in an *in vitro* model. In addition, there is a lack of interaction with astrocytes, pericytes and the basal lamina. However, this static *in vitro* model is widely used and has significantly enhanced understanding of BBB function. In the current study, experiments were controlled using FDX to monitor the paracellular transport in each of the control and treatment groups. Moreover, the Transwell® model is an excellent model for our studies as it allows for the determination of the mechanism of fatty acid transport across HBMEC by manipulation of the fatty acid transporters on the HBMEC.

There have been few studies that have examined the mechanism of fatty acid transport across the BBB. Edmond *et al* (1998, 2001) and Rapoport *et al* (2001) demonstrated the appearance of radiolabeled fatty acids in the brain after infusion into the blood but the mechanism of transport was not examined. Hamilton and Brunaldi (2007) postulated the existence of a passive diffusion model in which fatty acids cross the BBB using a reversible flip-flop mechanism. Although we cannot rule out some diffusion mediated transport of oleate across HBMEC, our data clearly indicates that a protein acceptor is a requirement for the transport of fatty acids across HBMEC of the BBB. In addition, transport of fatty acids across HBMEC appears to be a transcellular process mediated, in part, through a number of fatty acid transport proteins, FATP-1, FATP-4, CD36 and FABP5. Finally, fatty acids may move in either direction across HBMEC and this is mediated, in part, by these fatty acid transport proteins but there appears to be a

preference for oleate movement from apical to basolateral side.

We also examined if altering the level of FATP-1 in HEK 293 cells could regulate CL biosynthesis. Incorporation of [1,3-^{3}H]glycerol into CL was increased when FATP-1 was knocked down in HEK 293 cells compared to control. In addition, CL mass was elevated 2-fold when FATP-1 was knocked down in these cells. The reason for the increased CL content was likely an increase in [1,3-^{3}H]glycerol uptake and utilization for CL synthesis and an increase in activity and expression of the CL biosynthetic enzymes. The alteration in CL synthesis was reversed in FATP-1 expressing cells. In this case, [1,3-^{3}H]glycerol incorporation into CL was reduced in cells expressing FATP-1 compared to control even though [1,3-^{3}H]glycerol uptake was unaltered. The reason for the decrease in [1,3-^{3}H]glycerol incorporation into CL was a reduction in CDS enzyme activity and CDS-2 mRNA expression. Interestingly, the content of CL was unaltered in FATP-1 expressing cells compared to controls. Although *de novo* CL synthesis from [1,3-^{3}H]glycerol was lower in these cells expressing FATP-1, it was not surprising that there was no alteration in CL mass since normal turnover of CL is slow, in the matter of days, in mammalian cells (reviewed in Hostetler, 1982). The above observations indicate that CL synthesis is specifically modulated by FATP-1 knock down or expression.

Knock down of FATP-1 increased whereas FATP-1 expression did not affect PGPS and CLS activities. CDS is a rate limiting step of CL biosynthesis in the CDP-DG pathway

of mammalian cells (Hatch and McClarty, 1996). CDS activity was increased by FATP-1 knockdown and decreased by FATP-1 expression in HEK 293 cells. These alterations in CDS enzyme activity by alteration in FATP-1 expression suggest that the modulation of CL biosynthesis by FATP-1 expression is likely mediated through CDS. The reason for the increased CDS activity in FATP-1 siRNA transfected cells was an increase in CDS-2 mRNA expression. In addition, CDS-2 mRNA expression was reduced in FATP-1 expressing cells that exhibited a reduction in CDS activity. CDS-2 mRNA expression is widespread throughout the body with highest expression in the heart, brain and kidney (Heacock *et al*, 1996; Weeks *et al*, 1997; Saito *et al*, 1997; Halford *et al*, 1998; Volta *et al*, 1999). In contrast, CDS-1 mRNA expression is low in most tissues throughout the body outside of the retina (Heacock *et al*, 1996; Weeks *et al*, 1997; Saito *et al*, 1997; Halford *et al*, 1998; Volta *et al*, 1999). The above data suggest that CL biosynthesis may be modulated by FATP-1 expression through CDS activity and CDS-2 mRNA expression.

How does FATP-1 expression modulate CL biosynthesis through CDS? In addition to its fatty acid transport activity, FATP-1 is an acyl-Coezyme A synthetase that couples fatty acid synthesis to acyl-Coenzyme A production (Hall *et al*, 2003). CDS enzyme activity was shown to be inhibited by oleic acid in isolated hog lymph node lymphocytes (Scribney and Hegadorn, 1982). We observed a reduction in CDS activity with addition of exogenous oleoyl-Coenzyme A, indicating that elevation in fatty acid, previously observed in HEK 293 cells expressing FATP-1 (Hatch *et al*, 2002), may

inhibit CDS enzyme activity in these cells. Thus, the decrease in CDS activity observed in FATP-1 expressing cells could be due in part to an oleoyl-Coenzyme A accumulation in these cells. However, the free cytosolic concentration of acyl-Coenzyme A esters is in the low nanomolar range, and unlikely exceeds 0.2 μM under the most extreme conditions (Faergerman and Knudsen, 1997). This, it is likely that under normal physiological conditions CDS activity is not regulated by acyl-Coenzyme A levels. In addition, CDS activity was shown to be regulated by the composition of PA (Bishop and Strickland, 1976). For example, in rat brain membrane fractions a 20% reduction in CDS activity was observed when 1-stearoyl-2-oleoyl-PA was replaced with 1,2-dioleoyl-PA. Thus, increasing the oleate concentration of the PA substrate may alter CDS activity through substrate composition. We did not observe significant changes in the fatty acyl composition of PA in our experiments (data not shown). Thus, regulation of CDS activity through PA seems unlikely. It is well documented that fatty acids have a significant effect on RNA abundance of genes encoding proteins involved in fatty acid metabolism in mammals (Black *et al*, 2000). Fatty acids may increase the expression of fatty acid transport proteins including carnitine palmitoyl transferase-1 and FATP (Sessler and Ntambi, 1998). Hepatocyte nuclear factor-4α (HNF-4α) is a member of a transcription factor family involved in hepatocyte differentiation and cellular metabolism (Duncan *et al*, 1998; Fraser *et al*, 1998). HNF-4α is broadly expressed with very high mRNA expression in kidney (Kim *et al*, 2001). Expression of HNF-4α may be regulated by binding of long-chain acyl-Coenzyme A's and coexpression of acyl-Coenzyme A synthetase potentiated activation of HNF-4α (Hertz *et al*, 1998).

Transcription factor binding analysis of the CDS gene using PROMO predicts a potential HNF-4α binding site. Since FATP-1 is an acyl-Coenzyme A synthetase (Hall *et al*, 2003) alteration in expression of FATP-1 could potentially modulate HNF-4α activation and hence CDS expression. This will form the basis for future studies on the nature of regulation of CDS expression mediated by FATP-1. In summary, altering FATP-1 levels in HEK 293 cells appears to modulate CL synthesis via regulation through CDS activity and expression.

5 CONCLUSION

The conclusions derived from the data presented in this thesis:

1. Fatty acid transport across HBMEC is enhanced by the presence of a protein acceptor. Albumin did not have to be devoid of fatty acid in order to facilitate fatty acid transport.
2. There is a net influx of oleate into the brain from the blood. The transport of fatty acids into the brain appears to be of higher importance than the export of fatty acids into the blood.
3. The saturation of fatty acids and the fatty acid chain length alter the transport and passage of fatty acids across the HBMEC monolayer.
4. FATP-1, FATP-4, FABP5 are highly expressed in HBMEC, capillary-enriched mouse brain fraction as well as in the grey matter of human brain tissue.
5. Knockdown of FATP-1, FATP-4 and FABP5 appear to be selective for long-chain fatty acid transport across HBMEC. Whereas, CD36 knockdown significantly reduced all fatty acids examined.
6. CL synthesis is specifically modulated by FATP-1 knock down or expression. CL biosynthesis may be modulated by FATP-1 expression through CDS activity and CDS-2 mRNA expression
7. The decrease in CDS activity observed in FATP-1 expressing cells could be due in part to an oleoyl-Coenzyme A accumulation in these cells.

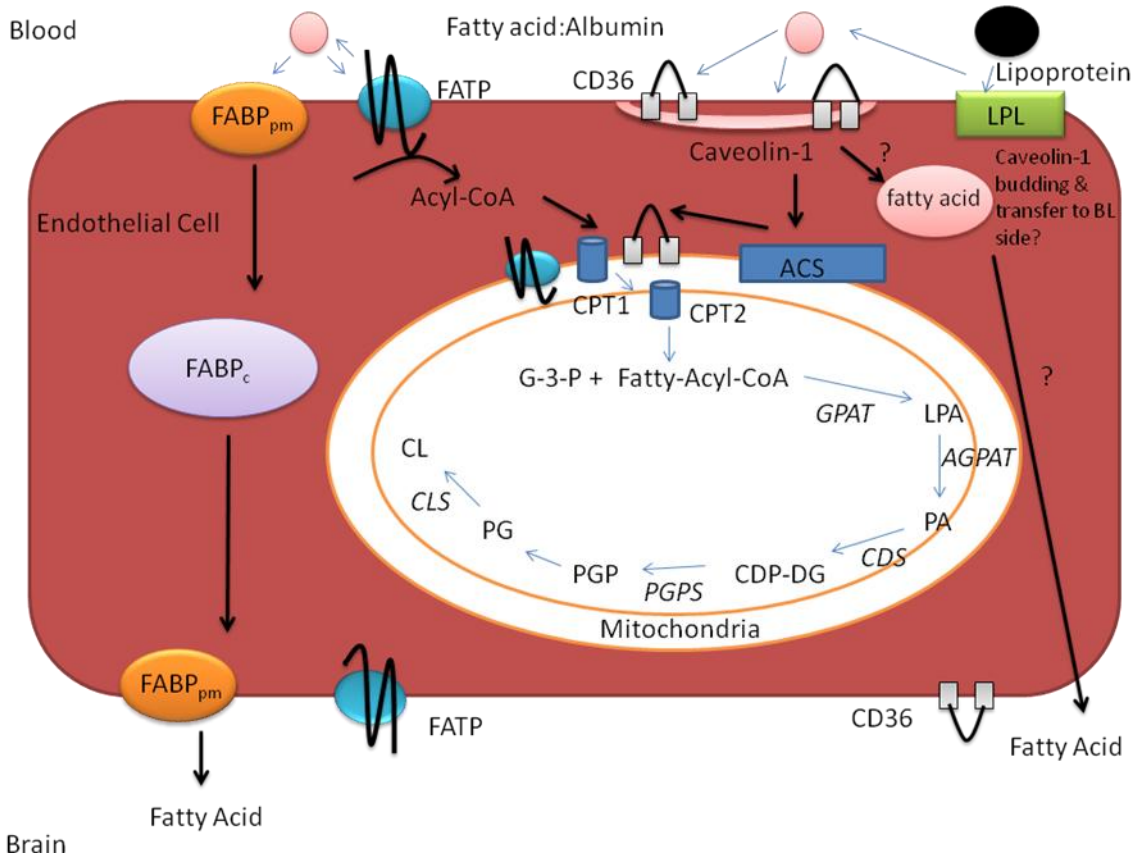

Figure 23

Summary. Proposed fatty acid transport across the endothelial cells of the BBB and the potential link to Cardiolipin biosynthesis from fatty acyl-CoA. G-3-P, glycerol-3 – phosphate; GPAT, glycerol-3-phosphate acyltranserase; AGPAT, acylglycerophosphate acyltransferase; LPA, Lysophosphatidic acid; PA, Phosphatidic Acid; CDS, Cytidylytransferase; PGPS, phosphatidyl glycerol phosphate synthase; PGP, phosphatidyl glycerol phosphate; PG, phosphatidyl glycerol; CLS, Cardiolipin synthase; CL, Cardiolipin. LPL, Lipoprotein Lipase; ACS, Acyl-CoA Synthetase; CPT1 & CPT2, Carnitine palmitoyltransferase 1 & 2; BL, basolateral

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TABLES

Table 1

Metabolism of [1-^{14}C]oleate in HBMEC.

HBMEC were incubated with 0.1 mM [1-^{14}C]oleate bound to albumin (1:1 molar ratio) for 2 h. Radioactivity into the fatty acid fraction and that incorporated into phospholipids; Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinosital and sphingomyelin were determined. Results represents the mean ± S.D. of three experiments.

Phospholipid	HBMEC (dpm/mg protein)
Phosphatidylcholine	82685 ± 7154
Phosphatidylethanolamine	3888 ± 989
Phosphatidylserine	691 ± 141
Phosphatidylinosital	864 ± 92
Sphingomyelin	923 ± 283
Fatty acid	125981 ± 36228

Table 2

Expression of select genes for fatty acid transporters in HBMEC and mouse (Ms) brain samples

Total RNA isolated and mRNA expression of FATP-1, FATP-2, FATP-3, FATP-4, FATP-5, FATP-6, CD36, FABP5, FABP7, Pgp, GFAP and β-actin determined as described in Materials and methods. Data represents the mean of three dishes, represented as percent expression relative to β-actin (± SE[a])

	HBMEC	Ms capillary	Ms deCapillary
FATP-1	5.67 (2.52)	1.31 (0.463)	0.468 (0.155)
FATP-2	ND[b]	0.109 (0.061)	0.072 (0.023)
FATP-3	0.335 (0.114)	0.042 (0.012)	0.009 (0.002)
FATP-4	4.54 (0.571)	1.91 (0.422)	0.322 (0.092)
FATP-5	0.002 (0.001)	0.002 (0.001)	0.002 (0.001)
FATP-6	ND[b]	0.039 (0.021)	0.016 (0.011)
CD36	0.256 (0.035)	0.006 (0.002)	0.002 (0.001)
FABP5	0.904 (0.069)	1.56 (0.464)	0.622 (0.234)
FABP7	0.009 (0.007)	0.226 (0.082)	1.62 (0.584)
Pgp	-	0.474 (0.093)	0.131 (0.042)
GFAP	-	0.072 (0.011)	1.00 (0.244)

[a] SE, standard error
[b] ND, not detectable

Table 3

Expression of select genes for fatty acid transporters in white matter and grey matter human brain samples

Total RNA was isolated and mRNA expression of FATP-1, FATP-2, FATP-3, FATP-4, FATP-5, FATP-6, CD36, FABP5, FABP7, Pgp, GFAP and β-actin determined using real-time PCR as described in Materials and Methods. Data represents the mean of three dishes, represented as percent expression relative to β-actin (± SE[a])

	White Matter	Grey Matter
FATP-1	0.617 (0.103)	1.192 (0.176)
FATP-2	0.004 (0.002)	0.055 (0.022)
FATP-3	0.138 (0.023)	0.317 (0.077)
FATP-4	0.174 (0.035)	1.369 (0.149)
FATP-5	0.003 (0.001)	0.009 (0.003)
FATP-6	0.017 (0.003)	0.067 (0.009)
FAT/CD36	0.001 (0.002)	0.010 (0.008)
FABP5	0.491 (0.084)	0.505 (0.085)
FABP7	0.022 (0.005)	0.189 (0.039)
Pgp	0.002 (0.001)	0.019 (0.002)
GFAP	0.108 (0.071)	0.106 (0.010)

[a] SE, standard error

Table 4

Summary of fatty acid permeability across HBMEC incubated with siRNA for fatty acid transport proteins

HBMEC were transfected with FATP-1, FATP-3, FATP-4, CD36 or FABP5 siRNA for 48 h as described in Materials and Methods. HBMEC were incubated apically with 0.1 mM [1-^{14}C] or [1-^{3}H] labeled fatty acid bound to albumin (1:1 molar ratio) for 10 min and radioactivity incorporated into the basolateral medium determined.

Fatty Acid	FATP-1	FATP-3	FATP-4	CD36	FABP5
Saturated Fatty Acids					
Butyric Acid	-	ND[a]	ND	+	ND
Myristic Acid	-	-	-	+	-
Palmitic Acid	+	-	-	+	-
Lignoceric Acid	-	ND	ND	+	ND
Monounsaturated Fatty Acids					
Oleic Acid	+	-	-	+	+
Polyunsaturated Fatty Acids					
Arachidonic Acid	-	ND	ND	-	ND
Linoleic Acid	-	-	+	+	+

[a]ND, not determined

Table 5

[1,3-^{3}H]Glycerol incorporation into phospholipids in mock and FATP-1 siRNA transfected cells.

Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H]glycerol for 24 h and radioactivity incorporated into phospholipids determined as described in Materials and Methods. Data represents the mean $\pm$ S.D. of three dishes.*$p<0.05$.

Phospholipid	Mock	FATP-1 Knockdown
	(dpm/mg protein x 10^{6})	
Cardiolipin	1.7 $\pm$ 0.4	3.3 $\pm$ 0.6*
Phosphatidylglycerol	0.4 $\pm$ 0.1	1.1 $\pm$ 0.1*
Phosphatidylcholine	17.6 $\pm$ 3.9	37.6 $\pm$ 2.9*
Phosphatidylethanolamine	6.4 $\pm$ 1.5	13.7 $\pm$ 1.1*
Phosphatidylserine/Inositol	1.1 $\pm$ 0.3	2.0 $\pm$ 0.1*
Phosphatidic acid	1.1 $\pm$ 0.2	2.5 $\pm$ 0.4*
Total radioactivity	30.6 $\pm$ 0.7	62.1 $\pm$ 0.5*

Table 6

Enzyme activities in FATP-1 siRNA transfected cells or FATP-1 expressing cells incubated with oleate.

Mock and FATP-1 siRNA transfected cells (**A**) or Mock and FATP-1 expressing C8 cells (**B**) were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and the enzyme activites of the CDP-DG pathway determined as described in Materials and Methods. Data represents the mean ± S.D. of three dishes.*$p<0.05$.

A.

Enzyme	Mock (pmol/min/mg protein)	FATP-1 knockdown (pmol/min/mg protein)
CDS	23.9 ± 0.1	31.0 ± 0.6*
PGPS	157.1 ± 5.8	198.8 ± 3.7*
CLS	0.7 ± 0.3	2.4 ± 0.6*

B.

Enzyme	Mock (pmol/min/mg protein)	C8 (pmol/min/mg protein)
CDS	41.2 ± 6.1	25.5 ± 2.4*
PGP	162.8 ± 31.5	163.1 ± 6.9
CLS	1.0 ± 0.2	0.9 ± 0.2

Table 7

[1,3-^{3}H]Glycerol incorporation into phosholipids in C8 cells expressing FATP-1.

HEK 293 mock and C8 cells expressing FATP-1 were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and then incubated with 0.1 mM [1,3-^{3}H]glycerol for 24 h and radioactivity incorporated into phospholipids determined as described in Materials and Methods. Data represents the mean $\pm$ S.D. of three dishes.*$p<0.05$.

Phospholipid	Mock	C8
	(dpm/mg protein x 10^5)	
Cardiolipin	3.5 $\pm$ 1.4	1.2 $\pm$ 0.4*
Phosphatidylglycerol	2.4 $\pm$ 0.2	1.7 $\pm$ 0.4*
Phosphatidylcholine	30.7 $\pm$ 8.4	12.4 $\pm$ 4.4*
Phosphatidylethanolamine	8.8 $\pm$ 1.9	5.1 $\pm$ 0.9
Phosphatidyserine	0.9 $\pm$ 0.2	0.6 $\pm$ 0.2
Phosphatidic Acid	0.6 $\pm$ 0.1	0.4 $\pm$ 0.3
Total Radioactivity	12.4 $\pm$ 0.1	11.1 $\pm$ 0.3

LEGENDS TO FIGURES

Figure 4. Percentage flux of FDX across Transwell® permeable inserts in the presence of HBMEC monolayers.

HBMEC were plated onto 6-well 0.4 µm Transwell® permeable inserts and incubated until confluent at 37^0C and 5.0% CO_2. Both blank inserts and HBMEC monolayers were incubated with 10 µM FDX (10,000 M) for up to 1 h. Basolateral medium was temporally sampled and the average percentage flux of FDX was determined. Data represents the mean $\pm$ S.D. of three experiments.

Figure 5. Percentage flux of FDX across HBMEC monolayer incubated with various concentrations of oleate.

HBMEC were incubated with 0-0.1 mM oleate bound to albumin (1:1 molar ratio) along with 10 µM FDX (10,000 MW) for up to 2 h. Basolateral medium was temporally sampled and the average percentage flux of FDX was determined. Data represents the mean $\pm$ S.D. of three experiments. Con, Control.

Figure 6. [1-^{14}C]oleate incorporation into basolateral medium in the presence of fatty acid free BSA.

HBMEC incubated with 0.1 mM [1-^{14}C]oleate bound 1:1 to albumin for up to 2 h. Total

radioactivity incorporated into the basolateral medium was determined in the presence or absence of fatty acid free BSA. Data represents the mean $\pm$ S.D. of three experiments. FAF, fatty acid free. *$p<0.05$ vs. no BSA; $^{\dagger}p<0.01$ vs. 30 min.

Figure 7. Directional permeability coefficients of oleic acid across HBMEC monolayers.

HBMEC were incubated with 0.1 mM [1-^{14}C]oleic acid bound 1:1 to albumin in the apical medium for up to 60 min and radioactivity incorporated into the basolateral medium determined. HBMEC were also incubated with 0.1 mM [1-^{14}C]oleic acid bound 1:1 to albumin in the basolateral medium for up to 60 min and radioactivity incorporated into the apical medium determined. A-B: apical to basolateral; B-A: basolateral to apical. Data represents the mean of three experiments.

Figure 8. Fatty acid permeability across HBMEC

HBMEC incubated with 0.1 mM [1-^{14}C]butyric acid or [1-^{14}C]myristic acid or [1-^{3}H]palmitic acid or [1-^{14}C]oleic acid or [1-^{14}C]linoleic acid or [1-^{3}H]arachidonic acid or [1-^{14}C]lignoceric acid bound 1:1 to albumin for up to 30 min. Total radioactivity incorporated into the basolateral medium was temporally determined. Data represents the mean $\pm$ S.D. of three experiments. Blue = unsaturated fatty acids; Red = saturated fatty acids.

Figure 9. The transport kinetics of [1-^{14}C]oleate uptake by HBMEC.

HBMEC were incubated apically with [1-^{14}C]oleate in the presence of various concentrations of unlabeled oleate (0μM -100μM) bound to albumin (1:1 molar ratio) for 2 h. Oleate concentrations are expressed on a log-scale. HBMEC were harvested after 2 h and the radioactivity incorporated in the cell extract was determined. Data represents the mean $\pm$ S.D. of three experiments. Inset; rate of oleate uptake by HBMEC.

Figure 10. [1-^{14}C]Oleate transport across HBMEC in the presence of 200μM phloretin.

HBMEC were incubated apically with 0.1 mM [1-^{14}C]oleate bound to albumin (1:1 molar ratio) in the absence or presence of 200 μM phloretin for up to 30 min. Total radioactivity incorporated into the basolateral medium was determined. Data represents the mean $\pm$ S.D. of three experiments. *$p < 0.05$.

Figure 11. FATP-1 protein expression in human brain tissue homogenates.

Human brain tissue protein samples, 1996c38 and 1996c39, (40 μg) were loaded onto a 7.5 % polyacrylamide gel and bands detected by enhanced chemiluminesence. FATP-1 protein expression determined by western blot analysis as described in Materials and

Methods. A representative blot is depicted.

Figure 12. FATP-1 and CD36 mRNA and protein expression, flux of FDX and [1-^{14}C]oleate transport in FATP-1 and CD36 siRNA transfected HBMEC.

HBMEC were transfected with FATP-1 or CD36 siRNA for 48 h as described in Materials and Methods. A. Total RNA was isolated and the % gene expression of FATP-1 and CD36 determined. B. Western blot of FATP-1 and CD36 mock and siRNA transfected HBMEC C. HBMEC were incubated with 10 μM FDX (10,000MW) for up to 20 min and the average percentage flux of FDX was determined in the basolateral medium. D. HBMEC were incubated with 0.1 mM [1-^{14}C]oleate bound to albumin (1:1 molar ratio) and radioactivity incorporated into the basolateral medium determined. Data represents the mean $\pm$ S.D. of three experiments. *$p<0.05$ vs. mock; **$p<0.001$ vs. mock; $^{\dagger}p<0.05$ vs. FATP-1

Figure 13. FATP-3, FATP-4 and FABP5 mRNA and protein expression in mock and siRNA transfected HBMEC.

HBMEC were transfected with FATP-3 or FATP-4 or FABP5 siRNA for 48 h as described in Materials and Methods. A. Total RNA was isolated and the % gene expression of FATP-1 and CD36 determined. B. Western blot of FATP-4 and FABP5 in

human brain tissue homogenates, mock and siRNA transfected HBMEC C. HBMEC were incubated with 10 μM FDX (10,000MW) for up to 30 min and the average percentage flux of FDX was determined in the basolateral medium. Data represents the mean ± S.D. of three experiments. *$p<0.05$ vs. mock

Figure 14. Fatty acid permeability across HBMEC incubated with siRNA for fatty acid transport proteins

HBMEC were transfected with FATP-1, FATP-3, FATP-4, FABP5 or CD36 siRNA for 48 h as described in Materials and Methods. HBMEC were incubated apically with 0.1 mM [1-^{14}C]butyric acid (A) or [1-^{14}C]myristic acid (B) or [1-^{3}H]palmitic acid (C) or [1-^{14}C]lignoceric acid (D) or [1-^{14}C]oleic acid (E) or [1-^{14}C]linoleic acid (F) bound to albumin (1:1 molar ratio) for 10 min and radioactivity incorporated into the basolateral medium determined. Data represents the mean ± S.D. of three experiments. *$p<0.05$ vs. mock; **$p<0.01$

Figure 15. Arachidonic acid increases permeability across HBMEC.

HBMEC were transfected with FATP-1 or CD36 siRNA for 48 h as described in Materials and Methods. A. HBMEC were incubated apically with 0.1 mM [1-^{3}H]arachidonic acid bound to albumin (1:1 molar ratio) for 30 min and radioactivity incorporated into the basolateral medium determined. B. HBMEC were incubated with

10 μM FDX (10,000MW) for up to 30 min and the average percentage flux of FDX was determined in the basolateral medium. Data represents the mean ± S.D. of three experiments.

Figure 16. [1-^{14}C]Oleate transport from basolateral to apical medium.

HBMEC were transfected with FATP-1 and CD36 siRNA for 48 h as described in Materials and Methods. Cells were incubated for up to 20 min with 0.1mM [1-^{14}C]oleate bound to albumin (1:1 molar ratio) in the basolateral medium and radioactivity incorporated into the apical medium determined. Data represents the mean ± S.D. of three experiments. *$p<0.01$ vs. mock; **$p<0.001$ vs. mock; $^{\dagger}p<0.01$ vs. FATP-1

Figure 17. FATP-1 mRNA expression in mock transfected HEK 293 cells and FATP-1 siRNA transfected cells.

Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA isolated and expression of FATP-1 and β-actin mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

Figure 18. Pool size of major glycerophospholipids in FATP-1 siRNA transfected cells

or FATP-1 expressing C8 cells incubated with oleate.

Mock and FATP-1 siRNA transfected cells (A) or mock and FATP-1 expressing C8 cells (B) were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and the pool size of the major glycerophospholipids were determined as described in Materials and methods. Data represents the mean $\pm$ S.D. of three dishes and is expressed as a percent of total phospholipid. *$p<0.05$.

Figure 19. CDS-2, PGPS and CLS mRNA expression in mock and FATP-1 siRNA transfected cells.

Mock and FATP-1 siRNA transfected cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA was isolated and expression of CDS-2 (A), PGPS (B) and CLS (C) mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

Figure 20. Protein expression profile in FATP-1 expressing cells.

Equivalent amounts of protein lysates from mock (A) and C8 (B) cells were separated by two-dimensional isoelectric focusing/sodium dodecyl sulphate polyacrylamide gel electrophoresis and stained as described in Materials and methods. Closed arrows in B

indicate a protein whose level was elevated; open arrows in B indicate proteins in which the level was reduced. A representative gel is depicted.

Figure 21. CDS-2 mRNA expression in mock and C8 cells expressing FATP-1.

Mock and C8 cells were incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h and total RNA isolated and expression of CDS-2 mRNA determined as described in Materials and methods. Data represents the mean of three dishes.

Figure 22. CDS activity in 293 cells in the presence of various concentrations of oleoyl-Coenzyme A.

(A) Mitochondrial fractions were prepared from HEK 293 cells and then incubated with 0 μM or 0.01 μM or 0.1 μM or 1 μM or 5 μM oleoyl-Coenzyme A and CDS activity determined as described in Materials and methods. (B) Long-chain acyl-Coenzyme A were determined in mock and C8 incubated with 0.1 mM oleate bound to albumin (1:1 molar ratio) for 24 h as described in Materials and methods. Data represents the mean $\pm$ S.D. of three dishes. *$p<0.05$, **$p<0.01$.

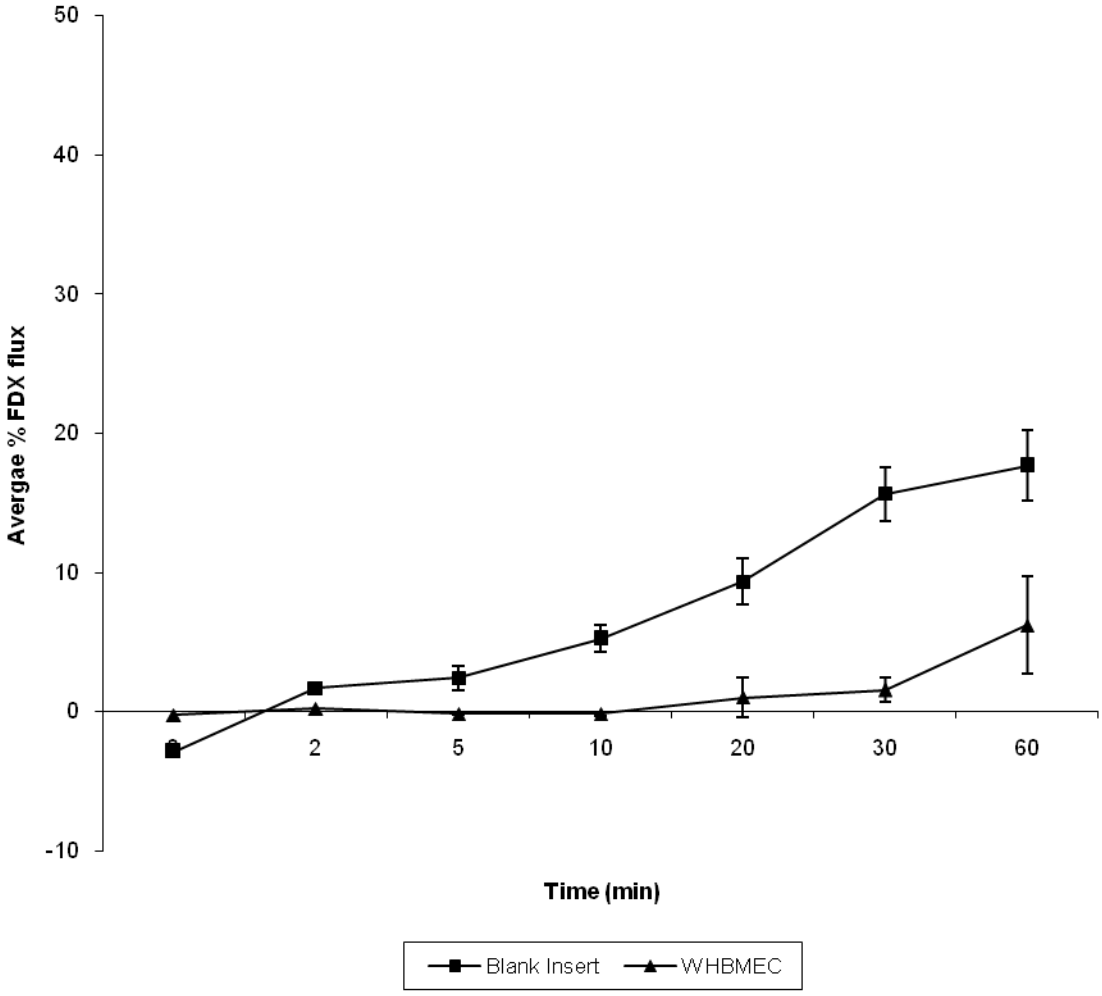

50
40
30
20
10
0
-10
Avergae % FDX flux
0
2
5
10
20
30
60
Time (min)
Blank Insert
WHBMEC

Figure 4

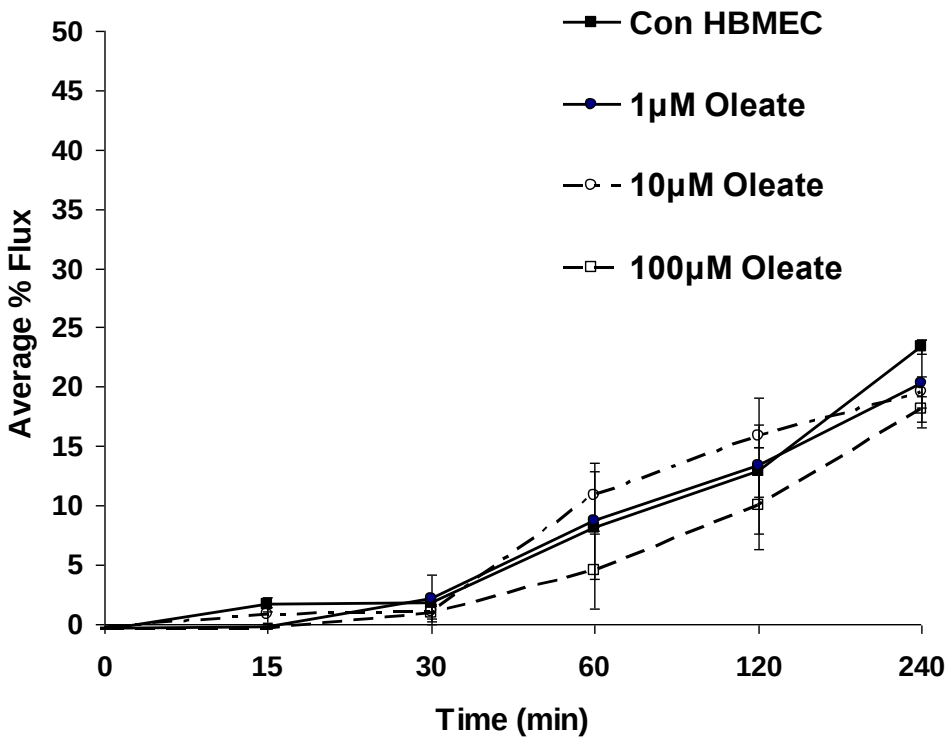

Con HBMEC
1µM Oleate
10µM Oleate
100µM Oleate
Average % Flux
50
45
40
35
30
25
20
15
10
5
0
0
15
30
60
120
240
Time (min)

Figure 5

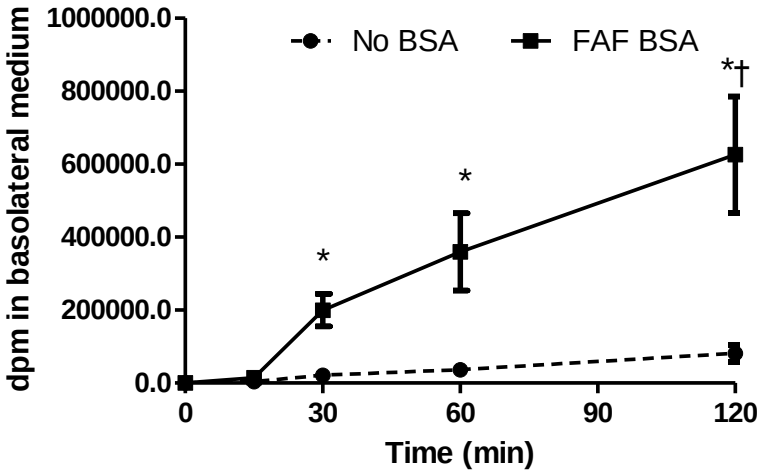

dpm in basolateral medium
1000000.0
800000.0
600000.0
400000.0
200000.0
0.0
No BSA
FAF BSA
*
*
*†
0
30
60
90
120
Time (min)

Figure 6

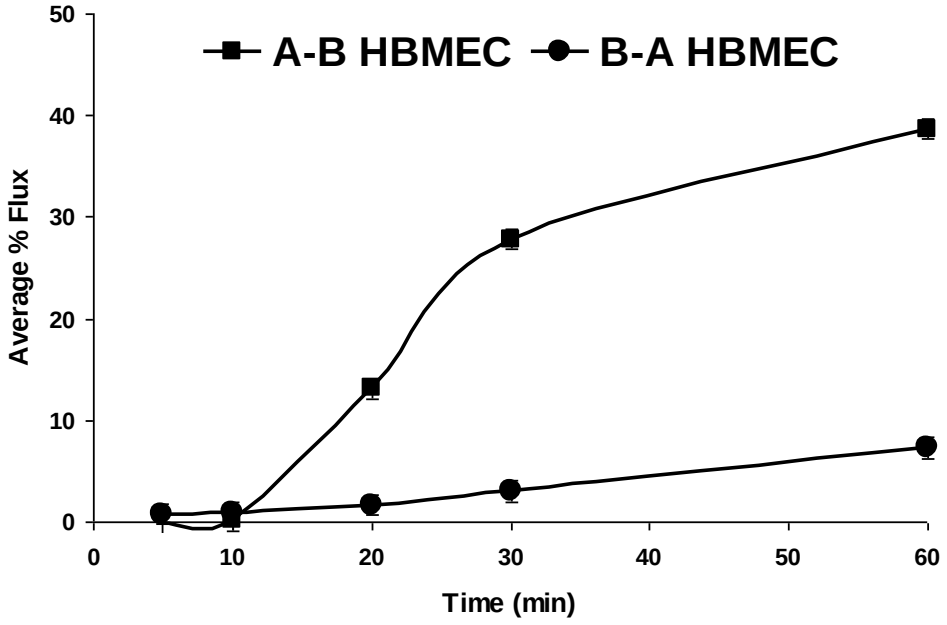


Figure 7

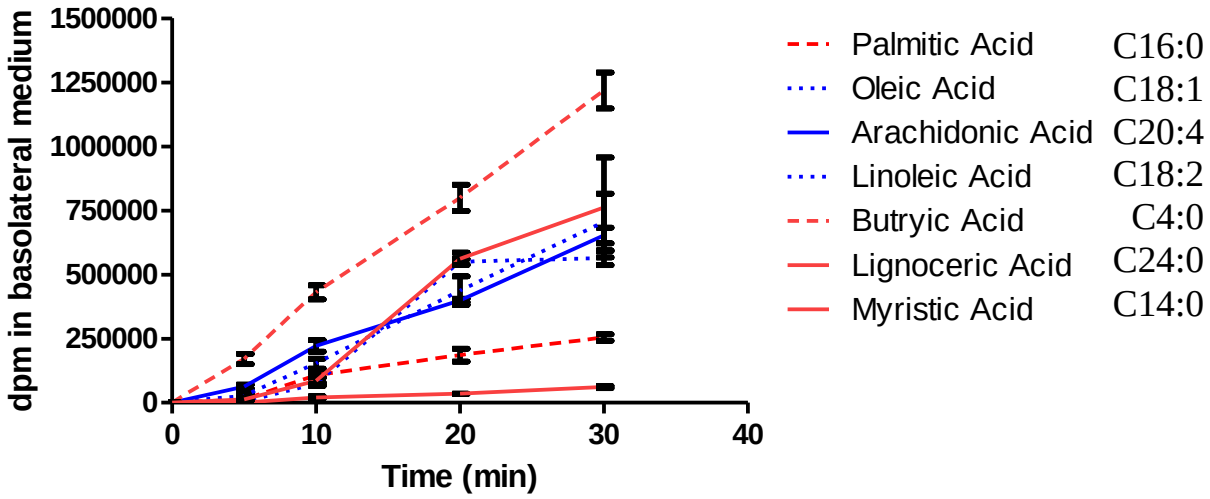

dpm in basolateral medium
1500000
1250000
1000000
750000
500000
250000
0
0
10
20
30
40
Time (min)
Palmitic Acid C16:0
Oleic Acid C18:1
Arachidonic Acid C20:4
Linoleic Acid C18:2
Butryic Acid C4:0
Lignoceric Acid C24:0
Myristic Acid C14:0

Figure 8

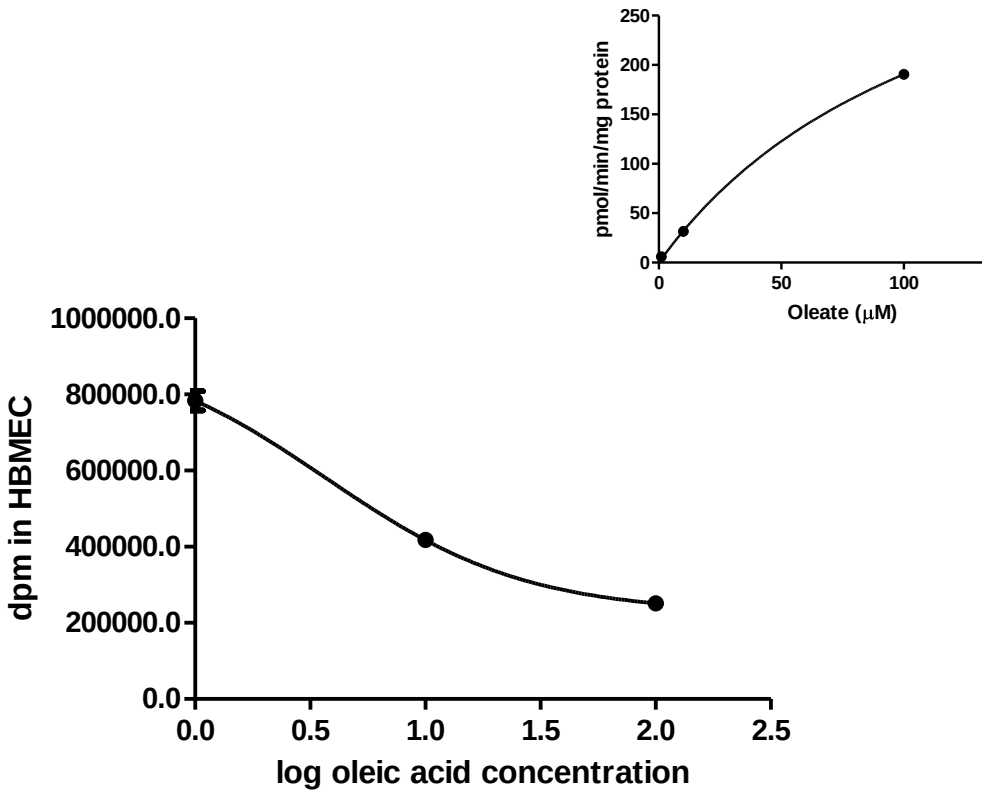

250
200
150
100
50
0
pmol/min/mg protein
0
50
100
Oleate (μM)
1000000.0
800000.0
600000.0
400000.0
200000.0
0.0
dpm in HBMEC
0.0
0.5
1.0
1.5
2.0
2.5
log oleic acid concentration

Figure 9

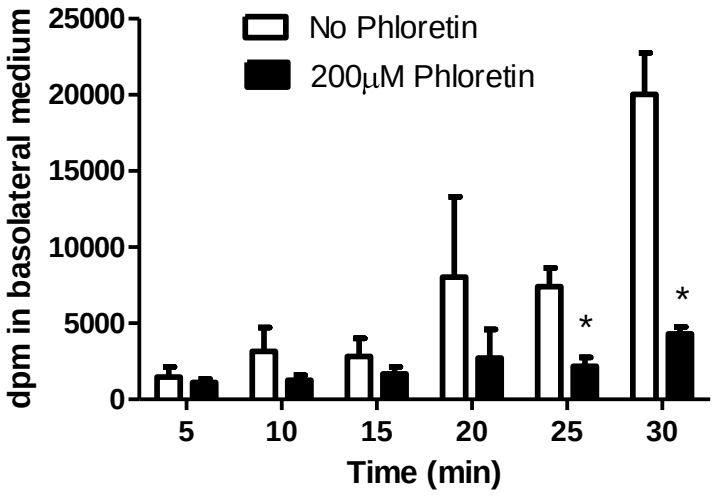

25000
20000
15000
10000
5000
0
dpm in basolateral medium
No Phloretin
200μM Phloretin
*
*
5
10
15
20
25
30
Time (min)

Figure 10

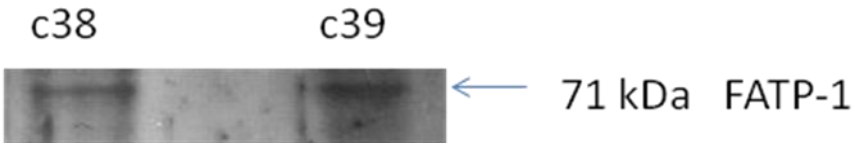


Figure 11

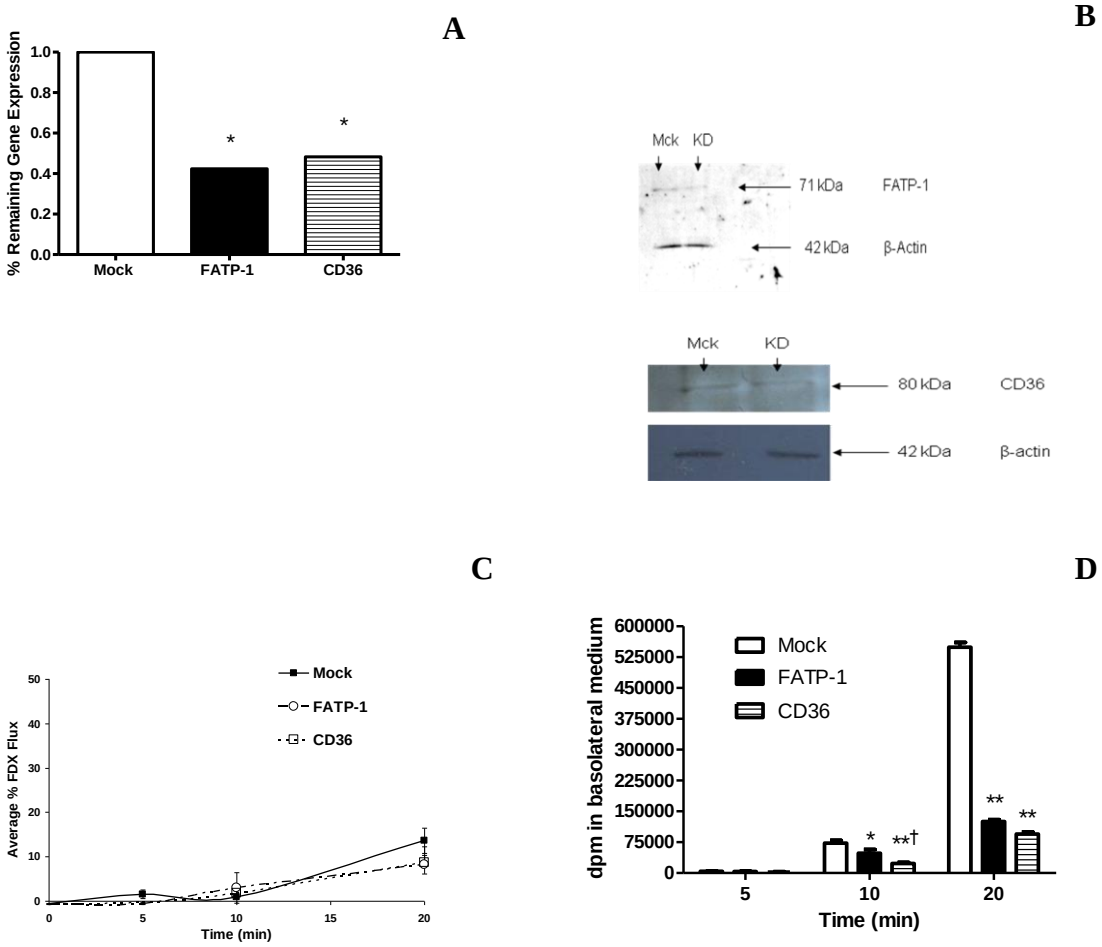
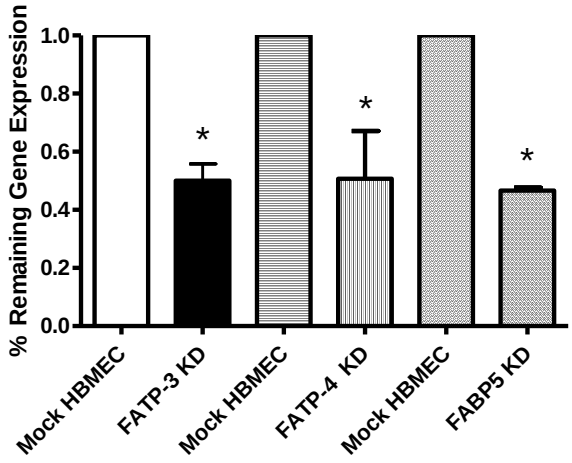
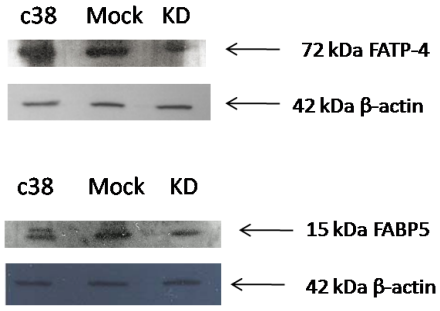

A
B
% Remaining Gene Expression
1.0
0.8
0.6
0.4
0.2
0.0
*
*
Mock
FATP-1
CD36
Mck KD
71 kDa FATP-1
42 kDa β-Actin
Mck KD
80 kDa CD36
42 kDa β-actin
C
D
Average % FDX Flux
50
40
30
20
10
0
0
5
10
15
20
Time (min)
Mock
FATP-1
CD36
dpm in basolateral medium
600000
525000
450000
375000
300000
225000
150000
75000
0
*
**†
**
**
5
10
20
Time (min)
Mock
FATP-1
CD36

Figure 12

A



B



C

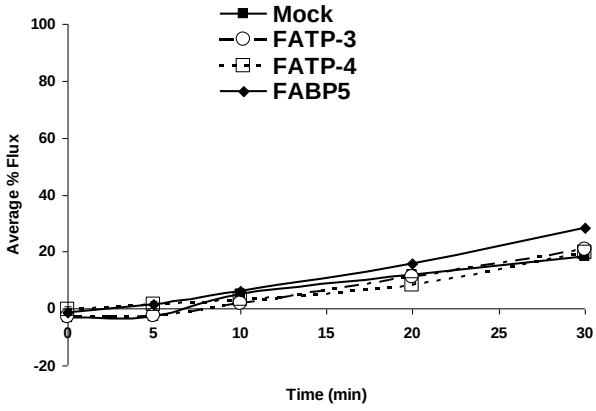


Figure 13

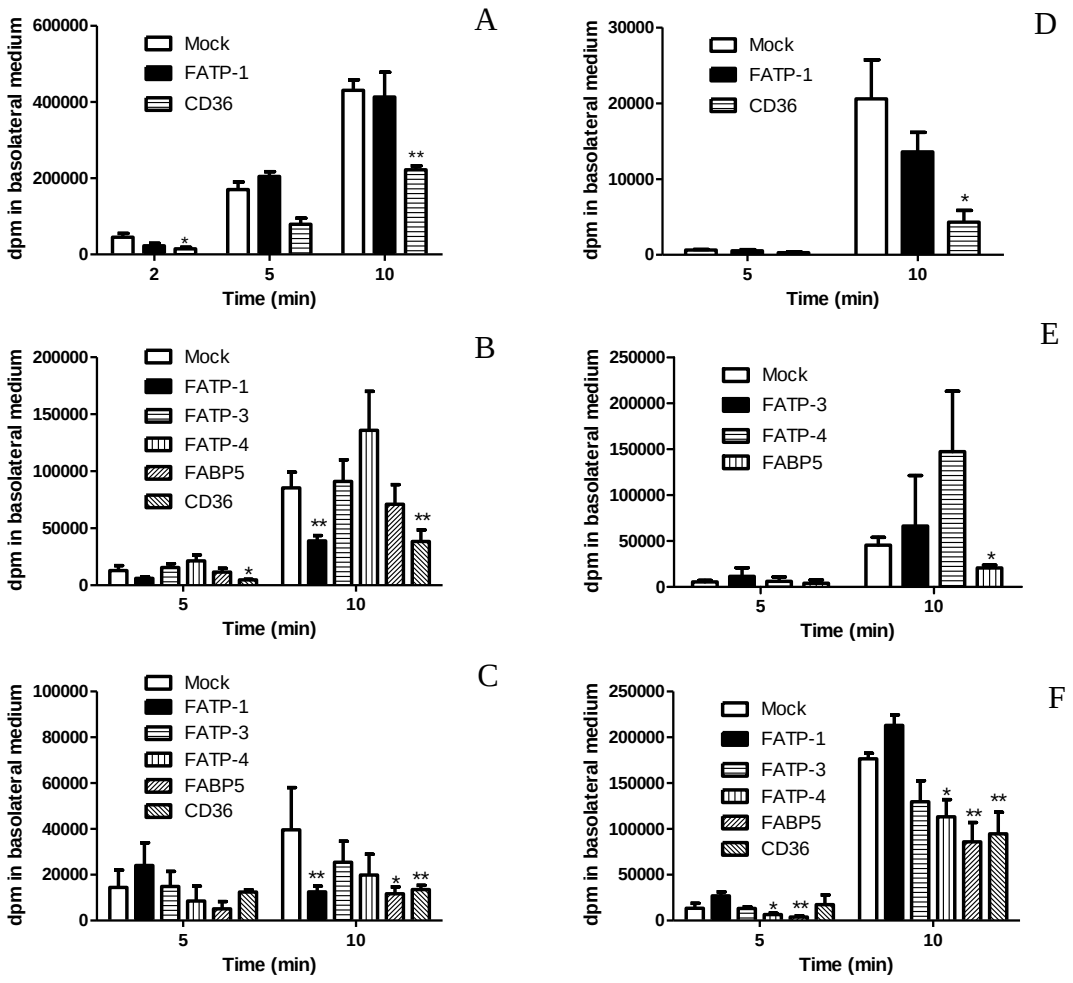
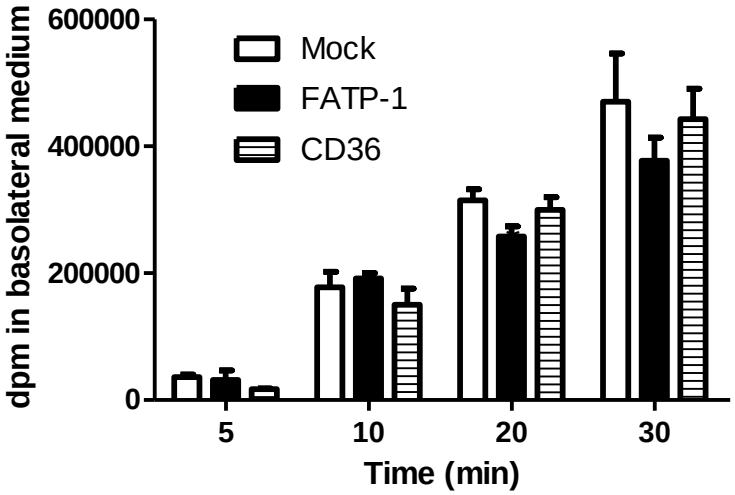

A
dpm in basolateral medium
600000
400000
200000
0
Mock
FATP-1
CD36
*
**
2
5
10
Time (min)
D
dpm in basolateral medium
30000
20000
10000
0
Mock
FATP-1
CD36
*
5
10
Time (min)
B
dpm in basolateral medium
200000
150000
100000
50000
0
Mock
FATP-1
FATP-3
FATP-4
FABP5
CD36
**
*
**
5
10
Time (min)
E
dpm in basolateral medium
250000
200000
150000
100000
50000
0
Mock
FATP-3
FATP-4
FABP5
*
5
10
Time (min)
C
dpm in basolateral medium
100000
80000
60000
40000
20000
0
Mock
FATP-1
FATP-3
FATP-4
FABP5
CD36
**
*
**
5
10
Time (min)
F
dpm in basolateral medium
250000
200000
150000
100000
50000
0
Mock
FATP-1
FATP-3
FATP-4
FABP5
CD36
*
**
*
**
5
10
Time (min)

Figure 14

A


dpm in basolateral medium
600000
400000
200000
0
Mock
FATP-1
CD36
5
10
20
30
Time (min)

B

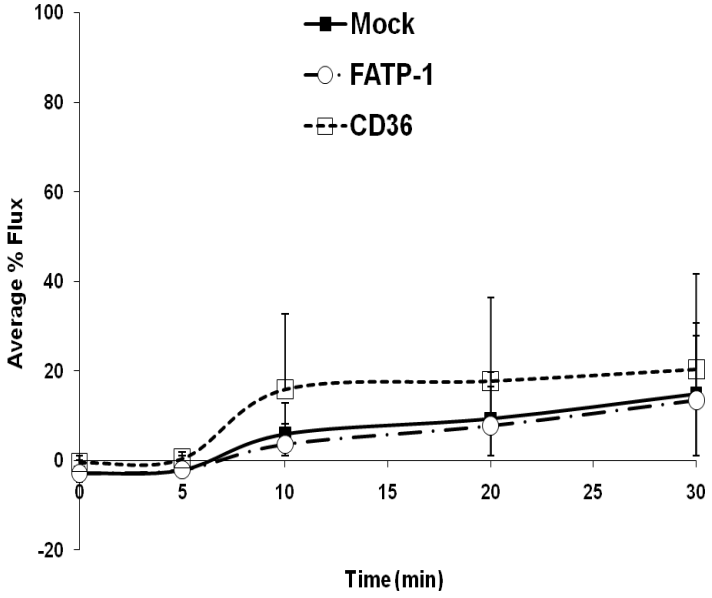

Mock
FATP-1
CD36
Average % Flux
100
80
60
40
20
0
-20
0
5
10
15
20
25
30
Time (min)

Figure 15

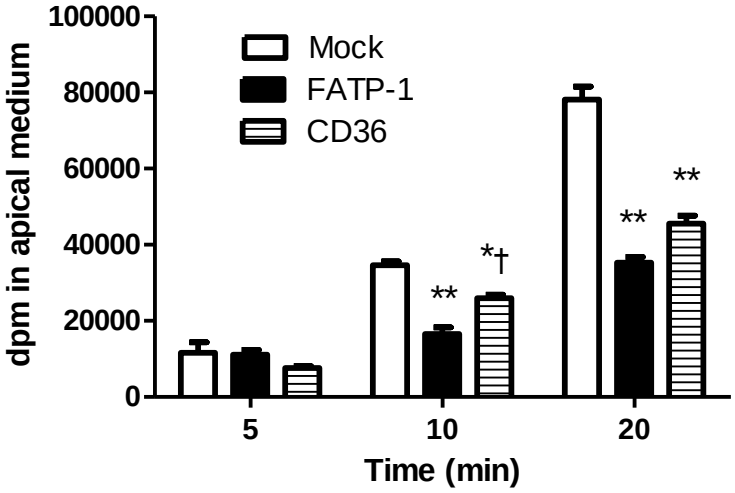

100000
80000
60000
40000
20000
0
dpm in apical medium
Mock
FATP-1
CD36
**
*†
**
**
5
10
20
Time (min)

Figure 16

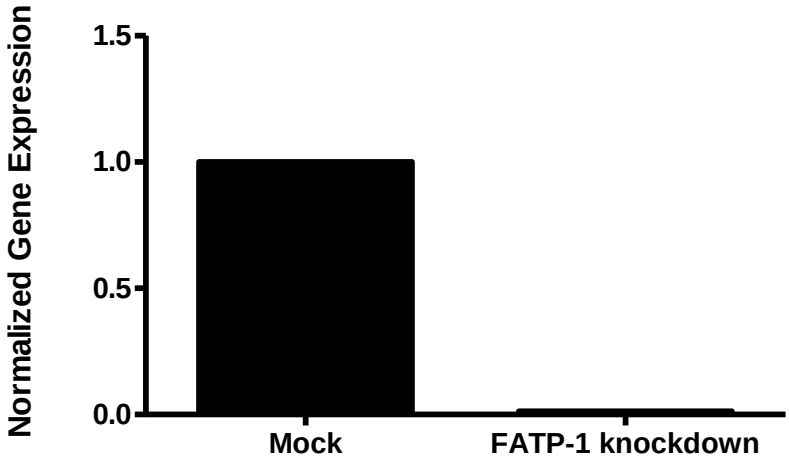
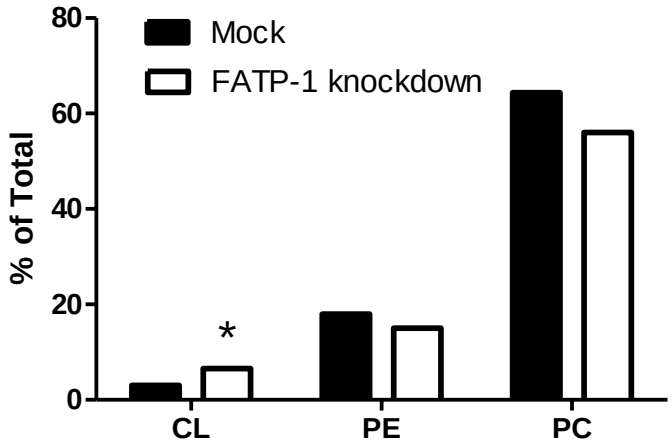

Normalized Gene Expression
1.5
1.0
0.5
0.0
Mock
FATP-1 knockdown

Figure 17

A


80
60
40
20
0
% of Total
Mock
FATP-1 knockdown
*
CL
PE
PC

B

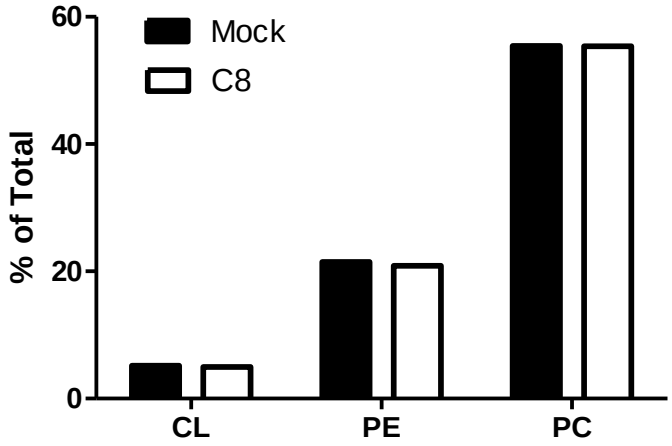

60
40
20
0
% of Total
Mock
C8
CL
PE
PC

Figure 18

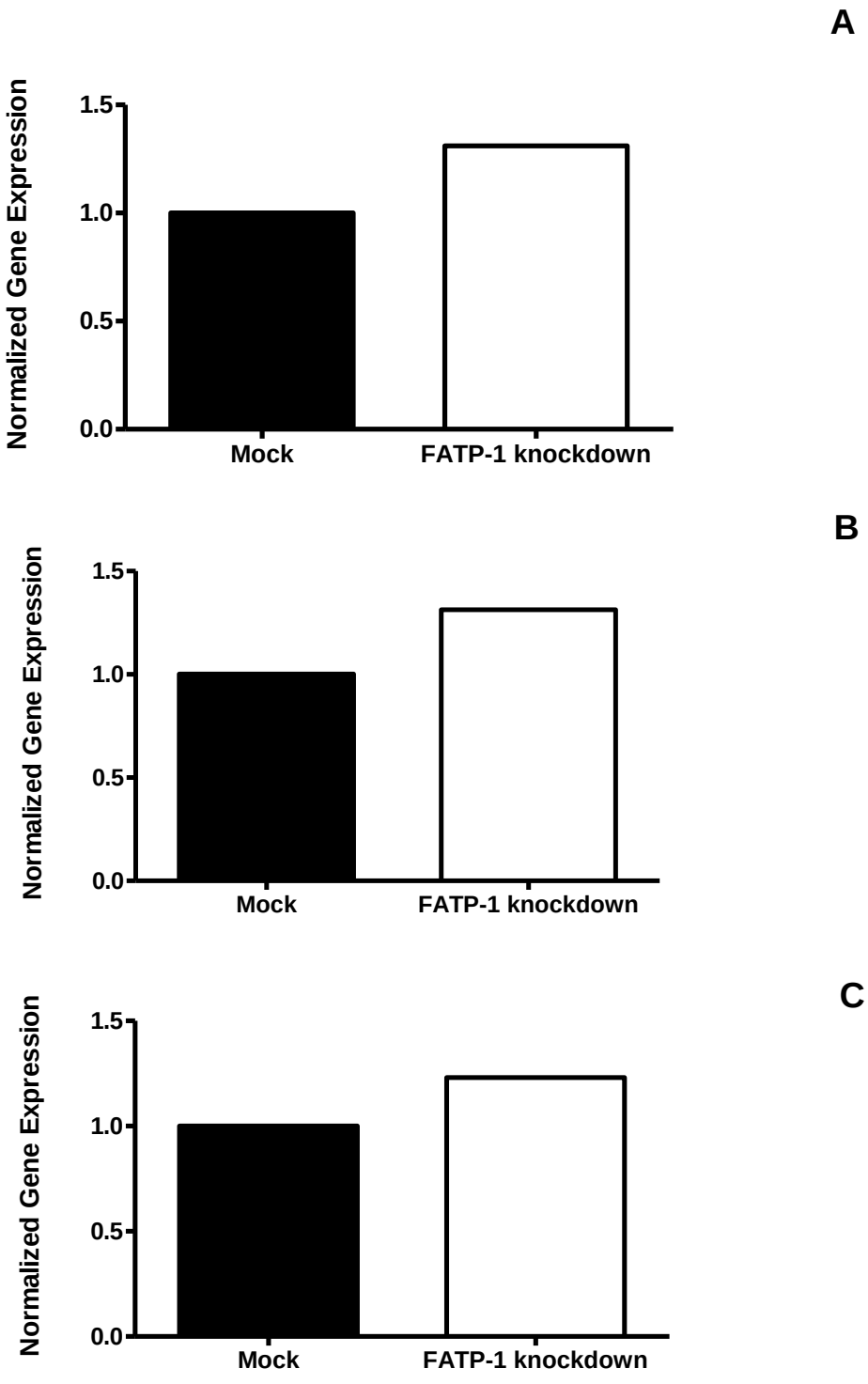

A
Normalized Gene Expression
1.5
1.0
0.5
0.0
Mock
FATP-1 knockdown
B
Normalized Gene Expression
1.5
1.0
0.5
0.0
Mock
FATP-1 knockdown
C
Normalized Gene Expression
1.5
1.0
0.5
0.0
Mock
FATP-1 knockdown

Figure 19

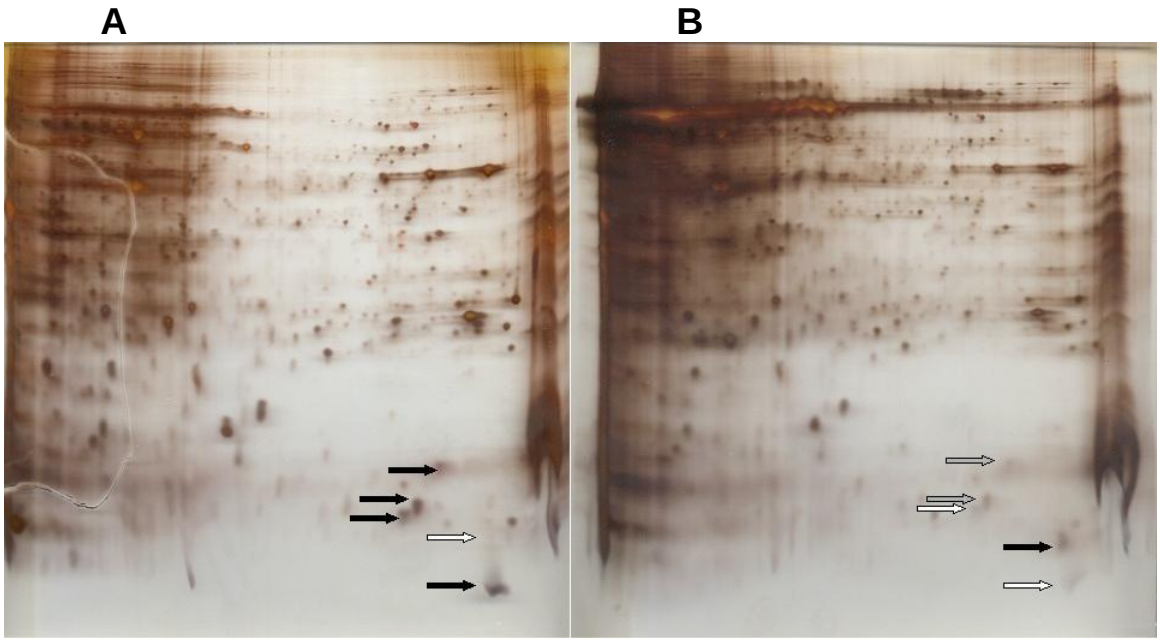


Figure 20

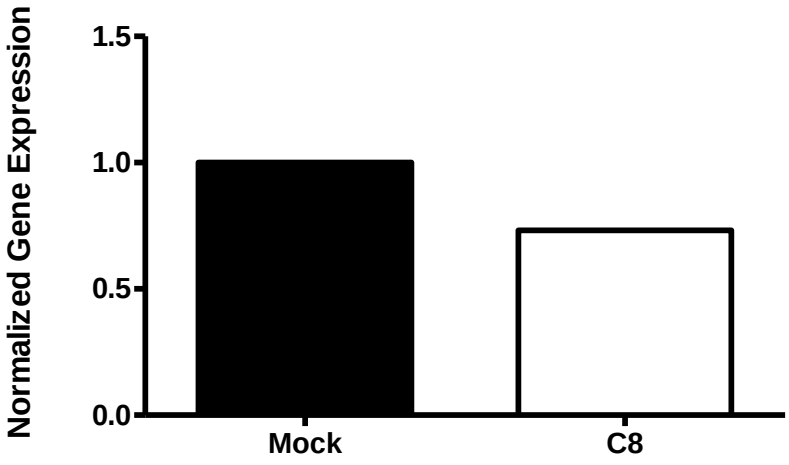


Figure 21

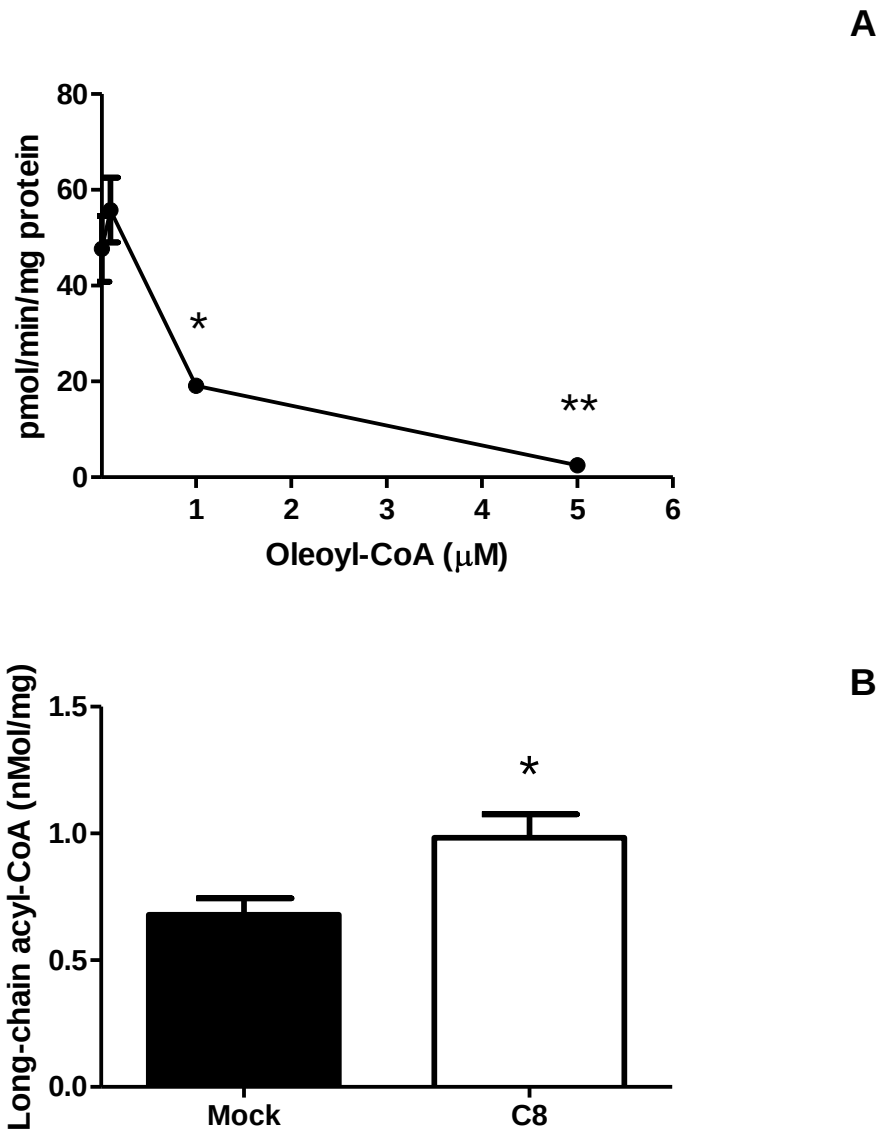

A
80
60
40
20
0
pmol/min/mg protein
*
**
1
2
3
4
5
6
Oleoyl-CoA (μM)
B
1.5
1.0
0.5
0.0
Long-chain acyl-CoA (nMol/mg)
*
Mock
C8

Figure 22