# Regulation of permeability of human brain microvessel endothelial cells by polyunsaturated fatty acids

by

Siddhartha Dalvi

A Thesis submitted to the Faculty of Graduate Studies

of

The University of Manitoba

in partial fulfillment of the requirements of the

degree of

**MASTER OF SCIENCE**

Department of Pharmacology and Therapeutics

University of Manitoba

Winnipeg

# ABSTRACT

The blood-brain barrier, formed by brain microvessel endothelial cells, is the restrictive barrier between the brain parenchyma and the circulating blood. It was previously demonstrated in our laboratory that knock down of fatty acid transport proteins FATP-1 and CD36 attenuated apical to basolateral monounsaturated fatty acid transport across human brain microvessel endothelial cells (HBMEC). Arachidonic acid (AA; 5,8,11,14 - *cis*-eicosatetraenoic acid) is a conditionally essential, polyunsaturated fatty acid [20:4(n-6)] and a major constituent of brain lipids. We examined transport of AA across confluent monolayers of HBMEC. Control cells or HBMEC with knock down of FATP-1 or CD36 were cultured on Transwell® plates and incubated apically with [$^3$H]AA and incorporation of [$^3$H]AA into the basolateral medium was determined temporally. [$^3$H]AA was rapidly incorporated into the basolateral medium with time in control cells. Surprisingly, knock down of FATP-1 or CD36 did not alter [$^3$H]AA movement into the basolateral medium. The increased permeability mediated by AA was likely caused by a metabolite of AA produced *de novo* and was confirmed by an increased movement of fluorescent dextran from apical to basolateral medium. HBMECs expressed $PGE_2$ synthase, cyclooxygenase-1 and -2, $PGE_2$ receptors, tight junction proteins and prostaglandin transporters. The AA-mediated increase in membrane permeability was not attenuated by cyclooxygenase inhibitor

drugs (NSAIDs). Incubation of the HBMEC monolayers with exogenous $PGE_2$ resulted in attenuation of the AA-mediated permeability increases. The results indicate that AA increases the permeability of the HBMEC monolayer likely via increased production of metabolites or by-products of the lipoxygenase or epoxygenase pathways. These observations may explain the rapid influx of AA into the brain previously observed upon plasma infusion with AA.

# ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Grant Hatch, for wholeheartedly supporting and encouraging me in all my endeavors beyond just my lab work or course work. Not even once did you say “no” to any of my ideas, suggestions or requests, and I am really very grateful to you for that.

I have a wonderful committee that has encouraged and helped me throughout. Thank you Dr. McNicol, Dr. Miller and Dr. Smyth, for believing in me and for always being there for me.

Our lab is a full house with many people and it was fun to work with Edgar and Hieu, my fellow graduate students, Dr. Laura Cole, our post-doctoral fellow and our staff, Marilyne, Bill and Fred, who were always eager to help.

I consider myself privileged to be a part of this wonderful Department of Pharmacology and Therapeutics. I would like to thank Dr. Parkinson, Dr. Bose, Dr. Fernyhough and Dr. Albensi, for making my journey very smooth and enjoyable, which I will cherish forever.

I also would like to thank three wonderful ladies, Karen Donald and Raquel Nowlin in Pharmacology and Debbie Korpesho at the Manitoba Institute of Child Health who have been wonderfully supportive and have gone out of their way to help me, always.

Finally, I have no words to thank my parents, my wife and my daughter, who have been my pillars of strength, and it is to them that I would like to dedicate this piece of work.

# TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# ABBREVIATIONS

| | |
|---|---|
| AA | Arachidonic acid |
| ABC | ATP-binding cassette |
| ACM | Astrocyte-conditioned medium |
| BBB | Blood-brain barrier |
| BBMEC | Bovine brain microvessel endothelial cells |
| BMEC | Brain microvessel endothelial cells |
| BSA | Bovine serum albumin |
| cAMP | Cyclic AMP |
| CLS | Cardiolipin synthase |
| CNS | Central nervous system |
| COX | Cyclooxygenase |
| cPGES | Cytosolic prostaglandin E synthase |
| $cPLA_2$ | Cytosolic phospholipase $A_2$ |
| CYP450 | Cytochrome P 450 |
| DHA | Docosahexaenoic acid |
| DHET | Dihydroxyeicosatrienoic acid |
| DP | Prostaglandin $D_2$ receptor |
| DPA | Docosapentaenoic acid |
| EAE | Experimental autoimmune encephalomylelitis |
| EET | Epoxyeicosatrienoic acid |
| eNOS | Endothelial nitric oxide synthase |

| | |
|---|---|
| EP | Prostaglandin $E_2$ receptor |
| EPA | Eicosapentaenoic acid |
| ER | Endoplasmic reticulum |
| FA | Fatty acid |
| FAT | Fatty acid translocase |
| FATP | Fatty acid transport protein |
| FDX | FITC-labeled dextran |
| FLAP | 5-lipoxygenase activating protein |
| FP | Prostaglandin $F_{2\alpha}$ receptor |
| GDNF | Glial cell-derived neurotrophic factor |
| GGT | Gamma-glutamyl transpeptidase |
| GLA | Gamma linolenic acid |
| GLUT-1 | Glucose transporter-1 |
| GM-CSF | Granulocyte- macrophage colony stimulating factor |
| GPCR | G protein- coupled receptor |
| HBMEC | Human brain microvessel endothelial cells |
| HDoHE | Hydroxydocosahexaenoic acid |
| HEETA | Hydroxyepoxyeicosatrienoic acid |
| HEPE | Hydroxyeicosapentaenoic acid |
| HETE | Hydroxyeicosatetraenoic acid |
| HPETE | Hydroperoxyeicosatetraenoic acid |
| IL | Interleukin |

| | |
|---|---|
| $iPLA_2$ | Calcium- independent phospholipase $A_2$ |
| JAM | Junctional adhesion molecule |
| LO | Lipoxygenase |
| LOX | Lipoxygenase |
| LPS | Lipopolysaccharide |
| LT | Leukotriene |
| $LTA_4H$ | Leukotriene $A_4$ hydroxylase |
| $LxA_4$ | Lipoxin $A_4$ |
| MAPEG | Membrane- associated proteins in eicosanoid and glutathione metabolism |
| MMP | Matrix metalloproteinase |
| mPGES | Membrane- bound prostaglandin E synthase |
| MRP | Multidrug resistance protein |
| NOS | Nitric oxide synthase |
| NSAIDs | Nonsteroidal anti-inflammatory drugs |
| OXO-ODE | Oxooctadecadienoic acid |
| PDGF | Platelet- derived growth factor |
| PES | Prostaglandin endoperoxide synthase |
| PG | Prostaglandin |
| PGDH | Prostaglandin dehydrogenase |
| PGES | Prostaglandin E synthase |
| PGHS | Prostaglandin H synthase |

| | |
|---|---|
| PGT | Prostaglandin transporter |
| PPAR | Peroxisome proliferator activated receptor |
| PUFA | Polyunsaturated fatty acid |
| RBC | Red blood cell |
| SPM | Specialized pro-resolving mediators |
| TEER | Trans- endothelial electrical resistance |
| TGF-β | Transforming growth factor- beta |
| THETA | Trihydroxyeicosatrienoic acid |
| TJ | Tight junctions |
| TNF-α | Tumor necrosis factor- alpha |
| TRAAK | TWIK- related arachidonic acid- stimulated $K^+$ channel |
| TREK | TWIK- related $K^+$ channel |
| TWIK | Twin pore inwardly rectifying $K^+$ channel |
| ZO | Zonula occludens |

# 1. BACKGROUND

## 1.1 Blood Brain Barrier

The blood brain barrier (BBB) is a selectively permeable cellular boundary between the brain and the peripheral circulation. The principal component of the BBB is the capillary or microvessel endothelial cell. The endothelial cells in the brain capillaries differ from those in the peripheral vasculature in several key features:

1. Presence of tight junctions (TJ) that limit the paracellular passage of macromolecules.
2. Restricted rate of fluid-phase endocytosis that limits the transcellular passage of macromolecules (Rubin *et al*. 1991)
3. Presence of specific transporter and carrier molecules (Gloor *et al*. 2001)
4. Lack of fenestrations (Hawkins and Davis 2005)
5. Increased mitochondrial content (Hawkins and Davis 2005)

Thus, the endothelial cells of the BBB are less “leaky” than those of the peripheral vessels. However, it has been shown that if the endothelial cells of the brain capillaries are removed from their natural environment and allowed to vascularize the peripheral tissue, they become leakier (Rubin *et al*. 1991). In contrast, the endothelial cells from the periphery form tight junctions when allowed to vascularize the brain parenchyma. Morphologically, the tight junctions of the BBB

resemble the tight junctions between epithelial cells rather than those between peripheral vascular endothelial cells (Kniesel and Wolburg 2000).

The unique tight junctions of the BBB are responsible for producing very high transendothelial electrical resistance (TEER) of 1500 – 2000 $\Omega cm^2$ (Mitchell *et al*. 2009; Mitchell *et al*. 2011; Gloor *et al*. 2001).

Though the microvessel endothelial cells play a primary role in the formation of the BBB, several other cells are equally important in maintaining the integrity of the BBB. These cells, namely, the astrocytes, pericytes, neurons and other glial cells are said to form a "neurovascular unit" (Urich *et al*. 2012).

Integrity of the BBB is of utmost importance in maintaining the homeostasis of the brain microenvironment. Disruption of the BBB is seen in various states of inflammation (multiple sclerosis), neoplasia, infections (meningitis, encephalitis), trauma and Alzheimer disease (Huber *et al*. 2001; Zlokovic *et al*. 2008). It would be highly desirable to develop therapeutic strategies to reverse this disruption and tighten the BBB. At the same time, a transient opening of the BBB would be advantageous for delivery of drugs into the brain in conditions like epilepsy or Parkinson disease (Gloor *et al*. 2001).

## 1.2 Functions of the BBB

The BBB is responsible for maintaining the appropriate ionic composition of the interstitial fluid of the brain that is required for optimum functioning of the neurons. To achieve this, the BBB functions as a **transport barrier** by facilitating the uptake of the required nutrients, while preventing the uptake of, or actively effluxing certain other molecules or toxic by-products of metabolism (Abbott *et al*. 2006).

The BBB also functions as a **metabolic barrier** by virtue of possessing intracellular and extracellular enzymes. For example, extracellular enzymes such as peptidases and nucleotidases break down peptides and ATP, respectively. Intracellular enzymes like cytochrome P450 (CYP450), primarily CYP1A and CYP2B degrade noxious substances and prevent their entry into the brain parenchyma (Abbott *et al*. 2006).

### 1.3 Role of astrocytes in the BBB

It is now known that the astrocytes play a key role in the conditioning and development of the brain microvessel endothelial cells (BMEC). Astrocytes are one of the glial cells of the central nervous system (CNS) that play several important roles in the structure and function of the CNS. They are intimately associated with the BMEC such that their foot processes ensheath 99% of the external surface of the

BMEC (Haseloff *et al*. 2005).

Astrocytes have been shown to alter the properties of co-cultured brain endothelial cells in the following ways: (Haseloff *et al*. 2005; Janzer and Raff 1987)

1. Increase in barrier-related marker enzyme activities, such as that of γ-glutamyl transpeptidase (GGT) and alkaline phosphatase.
2. Enhanced expression of glucose transporter GLUT1.
3. Elevation of trans-endothelial electrical resistance (TEER).
4. Tightening of the BBB as seen by decreased paracellular permeability of sucrose.
5. Increase in tight junction number, length and complexity.

It has also been shown that BMEC monolayers are less leaky if grown in the presence of astrocyte conditioned medium (ACM) (Rubin et al. 1991; Haseloff et al. 2005).

The precise molecular nature of the astrocyte-derived factors that is responsible for enhancing the barrier properties of the BBB has yet to be unequivocally elucidated. However, several factors have been postulated to play a role including glial cell-derived neurotrophic factor (GDNF), transforming growth factor-beta (TGF-β), and src-suppressed C-kinase substrate (SSeCKS) that leads to increased angiopoietin-1 secretion. The BMEC themselves are known to secrete factors that help in the maintenance of astrocyte health. One such

putative factor is leukemia-inhibitory factor (LIF), a cytokine known to be involved in astrocyte differentiation (Haseloff *et al*. 2005).

### 1.4 Role of pericytes in the BBB

The pericytes are specialized cells of mesenchymal lineage that have multiple organ-specific roles. For example, they are present in the kidney as mesangial cells, in the liver as perisinusoidal stellate cells and in the bone as osteoblasts (Balabanov and Dore-Duffy 1998; Lai and Kuo 2005). The pericytes in the central nervous system are closely associated with the BMEC and play an important role in the maintenance of the BBB. Their functions include (Lai and Kuo 2005):

1. Cerebrovascular autoregulation and blood flow distribution
2. Differentiation of the BBB
3. Formation and maintenance of the tight junctions of the BBB.
4. Initiation of the extrinsic (tissue factor) pathway of blood coagulation following cerebrovascular injury
5. Brain angiogenesis via secretion of angiopoietin-1
6. Phagocytic and scavenging (macrophage-like) functions
7. Production of immunoregulatory cytokines like IL-1β, IL-6 and GM-CSF
8. Regulation of leukocyte transmigration, antigen presentation and T-cell activation.

## 1.5 Molecular components of the tight junctions

The tight junctions consist of both membrane proteins as well as cytoplasmic proteins (Ballabh *et al*. 2004). The integral membrane proteins are:

- Claudins
- Occludin
- Junctional adhesion molecules (JAM)

There are also several cytoplasmic accessory proteins that form a plaque and function as adapter proteins to link the membrane proteins to the actin cytoskeleton of the cell (Matter and Balda 2003; Morita *et al*. 1999). These include:

- Zonula occludens proteins (ZO-1, ZO-2, ZO-3)
- Cingulin
- AF-6
- 7H6 antigen
- Symplekin

These tight junctional complexes are not static structures but rather very dynamic entities that can “bend without breaking”, thereby maintaining structural integrity (Huber *et al*. 2001).

## 1.6 Claudins

The claudins are a big family of transmembrane phosphoproteins

(Ballabh *et al*. 2004). Twenty four members have been characterized so far, claudins 1-24 (Sonoda *et al*. 1999; Krause *et al*. 2008). Of these, claudins 1, 3, 5 and 12 have been shown to form the tight junctions of the BBB (Furuse et al. 1998; Liebner et al. 2000; Morita et al. 1999, Zlokovic et al. 2008). Claudin-5 appears to be specific to the tight junctions of the endothelial cells and is called the “endothelial claudin” (Morita *et al*. 1999). Each claudin molecule has 4 transmembrane domains. The claudin on one cell binds homotypically to the claudin on the adjacent cell to form the seal of the tight junction. The claudins, along with occludin and the JAMs, form the tight junctional strands that keep the cells together and prevent paracellular flux of macromolecules from the apical to the basolateral side of polarized cells like BMEC (Sonoda *et al*. 1999). The cytoplasmic carboxy terminal of the claudins binds to the cytoplasmic ZO proteins (Furuse *et al*. 1998). Claudin-1 is an integral component of the tight junctions and its loss is associated with certain pathologic conditions like tumor, stroke and inflammatory diseases (Liebner *et al*. 2000).

### 1.7 Occludin

Occludin is a 65-kDa transmembrane phosphoprotein and is distinct from the claudins. However, its subcellular localization parallels that of claudins and, like the claudins, it has four transmembrane domains.

The expression of occludin is higher in the adult BMEC compared to the peripheral endothelial cells. However, it is not expressed in the fetal or newborn human brain. Occludin plays an important structural, as well as a functional, role in the regulation of BBB permeability. As is the case with several other tight junction-associated proteins, phosphorylation or dephosphorylation of serine, threonine or tyrosine residues on the occludin molecule is crucial for its proper functioning (Hirase *et al*. 1997; Sonoda *et al*. 1999; Morita *et al*. 1999; Feldman *et al*. 2005). For example, phosphorylation of occludin at serine and threonine residues correlates with tight junction assembly or tightening (Huber *et al*. 2001). At the same time, maintaining the tyrosine residues in a dephosphorylated state is also crucial for optimum interaction of occludin with ZO-1 (Rao 2009). The different protein kinases involved include c-Src, PKC zeta and PKC lambda/ iota. The different protein phosphatases involved include PP2A, PP1 and PTP1B (Rao 2009).

Occludin and the claudins interact intricately on the BMEC membrane. Together, they form channels that tightly regulate the paracellular flow of ions and other hydrophilic molecules. Thus, they are both essential in the formation, maintenance and regulation of the BBB (Sonoda *et al*. 1999; Matter and Balda 2003).

## 1.8 Junctional Adhesion Molecules (JAM)

These molecules play an important role in the regulation of tight junction permeability in endothelial and epithelial cells (Aurand-Lions *et al*. 2001). These glycoproteins are members of the immunoglobulin superfamily of proteins. Three different JAMs have been characterized in humans, JAM-1, JAM-2 and JAM-3, also referred to as JAM-A, JAM-B and JAM-C, respectively. Besides endothelial and epithelial cells, these molecules are also found on the surface of erythrocytes, leukocytes and platelets and are thought to contribute to various processes like leukocyte migration, platelet activation, angiogenesis and binding of reovirus (Mandell and Parkos 2005). The JAMs have short cytoplasmic tails that interact with cytoplasmic accessory proteins like ZO-1 and may require activation by phosphorylation, mediated by certain atypical protein kinases.

## 1.9 Cytoplasmic Accessory Proteins

Several cytoplasmic proteins appear to be essential components of the tight junctions. Among them, the **zonula occludens proteins** (ZO-1, ZO-2, ZO-3) play an important role. These 3 proteins have a molecular mass of 220, 160 and 130 kDa, respectively. They belong to a family of proteins called MAGUK (membrane-associated guanylate kinase-like protein) and form the submembranous plaque of the tight

junction (Ballabh *et al*. 2004; Gloor *et al*. 2001). They are structurally complex proteins with several domains that make direct contact with claudins, occludin and JAM on one side and the actin cytoskeleton on the other (Ballabh *et al*. 2004).

**Cingulin** is a double-stranded myosin-like protein that serves as scaffolding and links the TJ accessory proteins with the cytoskeleton (Huber *et al*. 2001).

**Actin**, the cytoskeletal protein, plays a central role in the maintenance of the TJ. Actin-degrading macromolecules, such as cytochalasin-B, cytochalasin-D, phalloidin and certain cytokines lead to disruption of the actin cytoskeleton and hence, of the tight junctions (Huber *et al*. 2001). It has been shown that increase in permeability of the BBB has direct correlation with the expression, depolymerization and spatial redistribution of actin filaments. Thickening of cortical actin correlates with reduction of paracellular permeability (Lai *et al*. 2005)

The tight junctional proteins can be modulated by several intracellular processes that involve calcium signaling, phosphorylation and G-proteins and extracellular factors like proteases and TNF-α (Huber *et al*. 2001; Kniesel and Wolburg 2000).

The tight junctional complexes also help localize the proteins and lipids of the apical and basolateral cell membranes in their respective

compartments and prevent free mixing of these cell membrane macromolecules between the two domains. Thus, the BMEC owe much of their polarity to the TJ complexes, as seen in electron microscopic and immunocytochemical studies on cerebral capillaries of stroke- prone spontaneously hypertensive rats (Gloor *et al*. 2001; Lippoldt *et al*. 2000).

### 1.10 Regulation of BBB permeability

To summarize, various factors play a role in regulating the permeability of the BBB as follows:

1. Post translational modifications of the TJ proteins. For example, phosphorylation and dephosphorylation mediated by protein kinases and phosphatases, respectively.
2. Alteration of the actin cytoskeleton.
3. Various stimuli such as infection, inflammation or ischemia give rise to enhanced expression and activity of metalloproteinases, especially MMP1, MMP3 and MMP9 (Verma *et al*. 2010). The MMPs ultimately degrade the tight junction proteins like occludin and claudin-5, that leads to a loosening of the BBB. This proteolysis and the resulting permeability increases can be prevented experimentally by pharmacological inhibitors of MMPs (Chiu and Lai 2013; Yang *et al*. 2007).

## 1.11 In vitro Models to Study the BBB

In vitro models of the BBB have proven very effective to study the transport of endogenous macromolecules like fatty acids across the BMEC. They have also been used extensively in pharmaceutical research to study the passage of therapeutic molecules across the BMEC (Mitchell *et al*. 2009; Mitchell *et al*. 2011; Urich *et al*. 2012). Several studies have shown that the BMEC lose many of their special properties when removed from their natural environment and show "dedifferentiation" behavior. Thus, one potential limitation of in vitro BBB models is that the BMEC may not behave as site-specific specialized brain endothelial cells in vitro, but rather as common peripheral endothelial cells (Urich *et al*. 2012). In spite of this shortcoming, several successful in vitro models of the BBB have been described (Gumbleton and Audus 2001). Many of these have used human, bovine, porcine or rat endothelial cells,

1. alone (Miller *et al*. 1992; Mitchell *et al*. 2009; Mitchell *et al*. 2011; Trickler *et al*. 2005; de Vries *et al*. 1996), or,
2. in combination with astrocyte - conditioned medium supplemented with agents that elevate intracellular cAMP (Rubin *et al*. 1991), or,
3. co-culture of endothelial cells on one side of a filter, with astrocytes on the other (Cecchelli *et al*. 1999).

## 1.12 Arachidonic Acid (AA)

Arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid) is one of the most important polyunsaturated fatty acids (PUFA) in the brain. Along with stearic acid, it is the most abundant fatty acid in brain phospholipids and comprises about 10% of the total fatty acid composition of the brain (Katsuki and Okuda 1995; McNamara and Carlson 2006). It is usually located at the sn-2 position of the glycerol backbone of membrane glycerophospholipids (Chan *et al*. 1985). It is released from glycerol by the action of phospholipase $A_2$ in response to various physiologic and pathologic stimuli. AA is a conditionally essential PUFA as it can be produced by elongation and desaturation of the essential fatty acid, linoleic acid or from gamma linolenic acid (GLA) in the liver (Rapoport 2008) and astrocytes (Moore *et al*. 1990). This helps maintain brain concentrations even on a diet devoid of arachidonic acid. However, in conditions of linoleic acid deficiency such as inadequate dietary intake, AA becomes an essential fatty acid (Burke *et al*. 1999).

About 4% of AA released from phospholipids is lost, either as various eicosanoids or by β oxidation in the mitochondria. However, the vast majority (96%) of AA released is recycled and reacylated in the brain (Rapoport 2008).

### 1.13 Physiologic (Neurotrophic) Roles of Arachidonic Acid

AA plays the following physiologic roles in the central nervous system:

1. AA is the precursor of eicosanoids (prostaglandins and leukotrienes)
2. It functions as a second messenger in cells by mobilizing calcium and activating $Ca^{2+}$ -dependent protein kinase C (Chan *et al*. 1985; Katsuki and Okuda 1995)
3. It enhances the activities of membrane-associated adenylate cyclase and guanylate cyclase to form cAMP and cGMP, respectively (Chan *et al*. 1985; Katsuki and Okuda 1995).
4. It stimulates ion transport ($Na^+$, $K^+$, $Cl^-$) across membranes like corneal epithelium (Chan *et al*. 1985; Katsuki and Okuda 1995)
5. It activates ion channels like mechanosensitive $K^+$ channels (TREK and TRAAK), $Ca^{2+}$ channels, gap junctions and dopamine transporter-associated cation current (Brash 2001)
6. It stimulates the release of amino acids and certain neurotransmitters like GABA from synaptosomes
7. It inhibits the activity of $Na^+$-$K^+$ ATPase in cell membranes
8. It activates NADPH oxidase (Brash 2001)
9. It regulates apoptosis in certain cells such as malignant tumor cells, while promoting survival in other cells. It also plays a role in neurite extension. It may, thus, play a pivotal role in the

developing CNS and in control of tumor development (Williams *et al*. 1998; Brash 2001)

10. Inhibition of inflammation
11. Nociception (Bosetti 2007)
12. Neuronal gene expression (Bosetti 2007)
13. Regulation of cerebral blood flow (Bosetti 2007)
14. Sleep / wake cycle
15. Appetite.

### 1.14 Pathologic (Neurotoxic) Roles of Arachidonic Acid

Most neurotoxic effects of AA are a result of increased formation of superoxide and other free radicals and consequent lipid peroxidation. AA plays the following pathological roles in the central nervous system:

1. AA is responsible for increases in BBB permeability and development of brain edema in response to pathologic stimuli like ischemia, trauma, infection or seizures (Chan *et al*. 1983; Chan *et al*. 1985).
2. Disturbances of AA metabolism are associated with disorders like Alzheimer disease and bipolar disorder when massive amounts of AA and other fatty acids are released (Rapoport 2008).
3. Altered AA metabolism has also been implicated in epilepsy,

ischemia, stroke, HIV-associated dementia, amyotrophic lateral sclerosis, Parkinson disease and schizophrenia (Katsuki and Okuda 1995; Bosetti 2007).

## 1.15 Metabolism of Arachidonic Acid

AA is metabolized to various biologically active substances by three different pathways:

1. Cyclooxygenase (COX) pathway: AA is converted to prostaglandins, prostacyclins and thromboxane $A_2$ (Bosetti 2007; Needleman 1986; Shimuzu and Wolf 1990; Arnold *et al*. 2010)
2. Lipoxygenase (LOX) pathway: AA is converted to leukotrienes, lipoxins, hepoxilins, hydroxyeicosatetraenoic acids (HETEs), trihydroxyeicosatrienoic acids (THETAs) and hydroxyepoxyeicosatrienoic acids (HEETAs) (Spector 2009; Gross *et al*. 2005; Smith and Murphy 2008).
3. Cytochrome P450 epoxygenase pathway: AA is converted to epoxyeicosatrienoic acids (EET), HETEs and hydroxyprostaglandins. The EETs are further hydrated to the corresponding dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase (Zeldin 2001; Kroetz and Zeldin 2002; McGiff 1991). Metabolism of AA by CYP enzymes is a significant source of oxygen free radicals in the vasculature (Roman 2002).

Besides being metabolized by these pathways, AA is also non-enzymatically autooxidized to isoprostanes.

## 1.16 Cyclooxygenases (COX)

The COX enzymes are also called prostaglandin endoperoxide synthases (PES) or prostaglandin H synthases (PGHS). The two cyclooxygenase isoforms, COX-1 and COX-2, mediate the conversion of arachidonic acid to $PGG_2$ and then to $PGH_2$ by catalyzing two reactions. The first reaction inserts two molecules of oxygen into arachidonate to form 15-hydroperoxy-9,11-endoperoxide with a substituted cyclopentane ring ($PGG_2$). The second reaction reduces $PGG_2$ to its 15-hydroxy analogue ($PGH_2$) (Needleman *et al*. 1986). Further conversion to specific eicosanoids is catalyzed by the different prostaglandin synthases (Dubois *et al*. 1998; Turini and Dubois 2002). The characteristics of the COX isoforms have been summarized in **Table 1.3**.

## 1.17 Cyclooxygenases and NSAIDs

The synthesis and secretion of PGs can be blocked by two therapeutically important classes of drugs, glucocorticoids and nonsteroidal anti-inflammatory drugs (NSAIDs). The glucocorticoids indirectly inhibit phospholipase $A_2$ via synthesis of lipocortin and thus

inhibit the release of AA from membrane phospholipids, the first step in the production of prostaglandins. The NSAIDs include drugs that non- specifically inhibit both isoforms of COX (aspirin, indomethacin, ibuprofen, naproxen) as well as specific inhibitors of COX-2 (celecoxib, rofecoxib, NS-398). Among the NSAIDs, aspirin is the only drug that irreversibly inactivates COX by acetylation of its active site at Ser530. The other NSAIDs inhibit COX reversibly by competing with AA for binding to COX. Though the non specific NSAIDs block both COX-1 and COX-2, it is the inhibition of COX-2 that is primarily responsible for their anti-inflammatory and analgesic actions (Smith and Murphy 2008).

## 1.18 Prostaglandins (PG)

The term “eicosanoids” refers to all the biomolecules produced by oxygenation of 20-carbon fatty acids, predominantly, arachidonic acid. Arachidonic acid (AA) is liberated from cell membrane phospholipids by the action of phospholipase $A_2$, primarily, the cytosolic type IV isoform, $cPLA_2$ and is the rate-limiting step in prostanoid synthesis (Bos *et al*. 2004; Funk 2001). The stimuli that trigger the cleavage of AA from membrane phospholipids include interaction of various biomolecules such as bradykinin, angiotensin II, thrombin, PDGF and IL-1 with their receptors (Smith and Murphy 2008). AA is further

converted to $PGG_2$ and then to $PGH_2$, which is the common precursor of several prostaglandins. This conversion is mediated by $PGH_2$ synthases, commonly referred to as cyclooxygenases (COX) (Yakubu *et al*. 2005). The two isoforms described, COX-1 and COX-2 are the targets of aspirin and the non steroidal anti-inflammatory drugs (NSAIDs).

$PGH_2$ is then converted by tissue-specific synthases into prostaglandins ($PGD_2$, $PGE_2$, $PGF_{2\alpha}$), prostacyclin ($PGI_2$) or thromboxane $A_2$ ($TXA_2$) by oxidative cyclization of the central 5 carbons within the 20-carbon fatty acid chain (Dubois *et al*. 1998). $PGE_2$ synthases are present in almost all tissues of the body, reflecting the ubiquitous synthesis of $PGE_2$. However, the other PGs have a relatively restricted distribution. $TXA_2$ synthase is predominantly present in platelets and macrophages. $PGI_2$ synthase is predominantly localized to endothelial cells. $PGF_{2\alpha}$ synthase is found in the uterus. $PGD_2$ synthase is found in the brain and mast cells (Funk 2001).

PGs are not preformed and stored in the cells as such, but are synthesized in response to specific stimuli (Smith *et al*. 1989). They function as local hormones and act in an autocrine or paracrine manner. To exert their effects, the PGs must first exit the cell of production and then act extracellularly on the same cell or

neighboring cells by means of cell surface receptors (Holla *et al*. 2008). Some PGs are also known to exert their effects by acting on nuclear PPAR receptors (PPAR α, γ, δ), which directly act as transcription factors upon binding with PGs (Dubois *et al*. 1998).

PGs are very potent biomolecules and their action has to be terminated in a timely manner to maintain tissue homeostasis. For termination of action, the PG molecules must be taken up by cells and then metabolized to inactive molecules (Holla *et al*. 2008; Reid *et al*. 2003). Several cell surface receptors and transporters mediate these functions, as discussed subsequently.

### 1.19 Prostaglandin E Synthases (PGES)

PGE synthases catalyze the conversion of $PGH_2$ to $PGE_2$ by isomerization. Three different isoforms have been described. They are designated cPGES (cytosolic PGES), mPGES-1 (membrane-bound PGES-1) and mPGES-2 (membrane-bound PGES-2). All the isoforms require glutathione (GSH) as an essential cofactor for activity (Helliwell *et al* 2003; Park *et al*. 2006). Development of drugs that inhibit PGES selectively could prove to be safer anti-inflammatory medications than the use of non selective COX or selective COX-2 inhibitors because of targeted therapy, with potentially fewer adverse effects (Park *et al*. 2006). The characteristics of the 3 isoforms have

been summarized in **Table 1.1**.

## 1.20 Prostaglandin $E_2$ ($PGE_2$)

$PGE_2$ is one of the most important and versatile PG and is the most abundant prostanoid in the human body (Park *et al*. 2006). $PGE_2$ produced within a cell is actively transported out of the cell by $MRP_4$, an efflux transporter of the ABC (ATP-binding cassette) family (Holla *et al*. 2008; Reid *et al*. 2003). $PGE_2$ then exerts its effects on cells by interacting with cell surface receptors. Four specific receptors have been described for $PGE_2$, designated as $EP_1$, $EP_2$, $EP_3$ and $EP_4$.

Termination of the action of $PGE_2$ is a two-step process. In the first step, $PGE_2$ is actively taken up by the PG transporter (PGT) in exchange for lactate. The $PGE_2$ is then oxidized to 15-keto $PGE_2$, an inactive product, by 15-hydroxy prostaglandin dehydrogenase (15 – PGDH) (Holla *et al*. 2008; Chan *et al*. 1997; Reid *et al*. 2003). $PGE_2$ is shown to have a proliferative effect on tumor cells and hence, 15-PGDH is considered to be a tumor suppressor as it causes reduction in $PGE_2$ levels (Smith and Murphy 2008). Further degradation involves reduction of the double bond between C-13 and C-14, ω-oxidation and β-oxidation.

## 1.21 $PGE_2$ Receptors

$PGE_2$, the most abundant PG in the body, exerts diverse and sometimes opposing effects in different tissues of the body. The reason for the multitude of effects lies in the localization and function of the $PGE_2$ receptors, through which the biological actions of $PGE_2$ are mediated. Four $PGE_2$ receptors have been characterized and are designated as $EP_1$, $EP_2$, $EP_3$ and $EP_4$ receptors (McCoy *et al*. 2002). These are present on the membranes of various cells in the body and are coupled to different second messenger systems, which explains their sometimes opposing effects. For example, $PGE_2$ can have both pro-inflammatory and anti-inflammatory effects (Park *et al*. 2006).

The EP receptors belong to the family of G protein-coupled receptors (GPCR), which are rhodopsin-like seven transmembrane spanning receptors that have 20-30% sequence similarity with each other (Negishi *et al*. 1995; Hata and Breyer 2004; Bos *et al*. 2004). Besides being present on the cell membrane, the EP receptors are also present within the nuclear membrane, where they act as transcription factors, notably for the synthesis of endothelial nitric oxide synthase (eNOS). The $EP_3$ receptor subtype appears to be the most abundant receptor subtype on the nuclear envelope, as seen in studies on porcine BMEC and on adult rat livers (Gobeil *et al* 2003; Bhattacharya *et al*. 1999; Helliwell *et al*. 2004). The characteristics of the EP receptor subtypes have been summarized in **Table 1.2**.

## 1.22 Multidrug Resistance Protein 4 (MRP4)

MRP4 belongs to the family of ABC transporters (ATP-binding cassette), subfamily C, and functions as an export pump to extrude $PGE_2$ and $PGE_1$ from their cell of production to the exterior. This step is necessary for the action of PGs (Reid *et al*. 2003; Park *et al*. 2006). The gene for MRP4 is located on chromosome 13q32.1 and encodes a protein of 1325 amino acids. The protein comprises 2 membrane-spanning domains, each consisting of 6 transmembrane helices and 2 ATP-binding cytosolic domains (Russel *et al*. 2008). The transporter utilizes ATP to pump $PGE_2$ against a concentration gradient. This pump is inhibited by indomethacin, suggesting that NSAIDs may also exert their anti-inflammatory effects by preventing the release of PGs from cells (Reid *et al*. 2003). The other members of the MRP family (MRP1, MRP2, MRP3 and MRP5) do not appear to be involved in $PGE_1$ or $PGE_2$ efflux. Besides being expressed on the apical surface of BMEC, MRP4 is also expressed on neurons, microglia and astrocytes in the CNS and on the parenchymal cells of the prostate, kidney, adrenals, lung, pancreas, testis and ovary (Dallas *et al*. 2006; Russel *et al*. 2008). Besides transporting $PGE_2$, MRP4 also effluxes a variety of endogenous and exogenous compounds. The endogenous compounds include molecules involved in cell signaling and communication, such as cyclic nucleotides, ADP, eicosanoids, and

conjugated steroid hormones as well as other metabolically important molecules such as folate, urate, bile acids and glutathione (Russel *et al*. 2008).

## 1.23 Prostaglandin Transporter (PGT)

The PGT is widely expressed on various cell types. It is responsible for the uptake of extracellular PGs within cells for the purpose of conversion to inactive metabolites as a means of terminating their actions. It is a 12-membrane-spanning domain integral membrane protein. It mediates specific and high affinity uptake of $PGE_2$ and $PGF_{2\alpha}$ and low affinity uptake of $PGD_2$, 8-iso-$PGF_{2\alpha}$ and $TXB_2$ (Schuster *et al*. 1998).

## 1.24 Leukotrienes

Leukotrienes are extremely potent, proinflammatory eicosanoids derived from AA via the 5-lipoxygenase (5-LO) pathway. They are synthesized predominantly in the inflammatory leukocytic cells like neutrophils, macrophages and mast cells, on stimulation by proinflammatory stimuli like immune complexes or infectious agents (Flamand *et al*. 2007). These stimuli lead to the liberation of AA by the action of $cPLA_2$. AA is converted to leukotriene $A_4$ ($LTA_4$) by the action of two enzymes acting in concert: 5-lipoxygenase (5-LO) and FLAP

(5-LO activating protein) (Peters-Golden and Henderson 2007). $LTA_4$ is converted by the cytosolic and/or nuclear enzyme $LTA_4$ hydrolase ($LTA_4H$) to $LTB_4$. On the other hand, $LTA_4$ can also be directly converted to $LTC_4$, one of the cysteinyl leukotrienes by conjugation with glutathione, which is catalyzed by the nuclear envelope enzyme, $LTC_4$ synthase, another member of the MAPEG family. $LTB_4$ and $LTC_4$ are then transported out of the cell by the efflux transporter MRP1. $LTC_4$ is converted extracellularly to $LTD_4$ and $LTE_4$, the other cysteinyl leukotrienes by successive elimination of the γ-glutamyl residue and glycine (Funk 2001; Samuelsson *et al*. 1987).

## 1.25 Leukotriene Receptors

The three cysteinyl leukotrienes, $LTC_4$, $LTD_4$ and $LTE_4$, exert their effects on target organs by interacting with specific cysteinyl LT receptors, $CysLT_1$ and $CysLT_2$. Similarly, two distinct receptors have been characterized for $LTB_4$, $BLT_1$ and $BLT_2$. The LT receptors belong to the A5 subfamily of the rhodopsin receptor-like, seven-transmembrane G protein-coupled receptors (GPCR) and are coupled to $G_{\alpha q}$ second messenger system that leads to an increase in intracellular $Ca^{2+}$ levels (Evans *et al* 2003; Back *et al*. 2011). The characteristics of the leukotriene receptor subtypes have been summarized in **Table 1.4**.

## 1.26 Pathophysiological Roles of Leukotrienes

The pathophysiological roles of $LTB_4$ can be summarized as follows (Flamand *et al*. 2007):

1. Neutrophil chemotaxis, leading to increased influx of inflammatory cells within tissues
2. Neutrophil adherence to vascular endothelium
3. Activation of neutrophils.

The pathophysiological roles of cysteinyl leukotrienes can be summarized as follows (Flamand *et al*. 2007):

1. Bronchoconstriction
2. Increased mucus secretion by the bronchial mucosa
3. Vasoconstriction of arteries and veins
4. Increased endothelial cell permeability causing edema of tissues
5. Attraction and activation of some leukocytes like eosinophils and monocytes.

## 1.27 Lipoxins

Lipoxins are lipid mediators derived from arachidonic acid by the lipoxygenase pathway. Their synthesis and activity is associated with the resolution phase of inflammation. This resolution phase is an active process that is characterized by eicosanoid “class switching”

from the prostaglandin and leukotriene pathway to the production of lipoxins (Serhan 2007). The lipoxins, along with the EPA and DHA metabolites resolvins, protectins and maresins, play an anti-inflammatory, pro-resolving and anti-fibrotic role in the resolution of inflammation, as well as protective roles in the nervous system, liver, lung and eye and in microbial clearance (Serhan 2005; Serhan and Chiang 2008). Lipoxins are synthesized from AA by the action of 5-LO and 15-LO in activated granulocytes. In the vasculature, lipoxin synthesis occurs by the interaction of 5-LO in myeloid cells and 12-LO in the platelets (Samuelsson *et al*. 1987; O'Meara *et al*. 2007).

### 1.28 Docosahexaenoic acid (DHA)

DHA [4,7,10,13,16,19-*cis*-docosahexaenoic acid, or, 22:6(n-3)] is one of the most abundant long chain PUFA present in the brain, retina and spermatozoa, where it is mainly esterified in membrane phospholipids (Picq *et al*. 2010). It comprises 10-20% of the total fatty acid composition of the brain (McNamara and Carlson 2006). Incorporation of DHA into brain phospholipids occurs at an especially fast rate in the developing brain. DHA, if not obtained directly from dietary sources, can be produced in the body from α- linolenic acid [18:3(n-3)], an essential fatty acid, through chain elongation and desaturation processes. The liver is the primary site for these

elongation and desaturation steps and the DHA thus produced is secreted into the blood for subsequent uptake by the brain.

Among the CNS cells, astrocytes are the only cells that posses the capacity of conversion of α- linolenic acid to DHA. Normally, this represents a minor source of DHA synthesis, especially when dietary DHA or that produced by the liver are adequate. However, astrocytes do play an important role in the uptake of DHA from the blood and its subsequent transfer to the neurons as part of its neurotrophic function (Kim 2007). Transport of DHA across the BBB, especially in the form of LysoPC-DHA, is of importance in maintaining adequate levels of DHA in the brain (Picq *et al*. 2010).

Neurons cannot completely convert α- linolenic acid to DHA due to lack of desaturase activity. BMEC can elongate and desaturate shorter chain fatty acids. However, they lack the Δ5 and Δ6 desaturases to produce DHA or docosapentaenoic acid (DPA).

DHA can be converted by lipoxygenases to its hydroperoxy and hydroxyl (HDoHE) derivatives (Picq *et al*. 2010). DHA is also a precursor in the formation of various anti-inflammatory compounds like resolvins, maresins, protectins and nitrolipids (Das 2010).

Loss of DHA from the brain is seen in aging and also in pathological conditions like Alzheimer disease and alcoholism (Kim 2007). Decreased concentration of long chain PUFA, both DHA and AA, are

seen in the plasma and red blood cell (RBC) membranes of children diagnosed with attention-deficit hyperactivity disorder (ADHD), autistic spectrum disorders and Asperger syndrome. Among these children, those with lower concentrations of omega-3 fatty acids had significantly more behavioral problems than those with higher concentrations (Bell *et al*. 2004; Burgess *et al*. 2000).

## 1.29 DHA Cycle

In the brain, DHA is concentrated in the growth cones, synaptosomes, astrocytes, myelin and mitochondrial and microsomal membranes. It is acylated into the sn-2 position of phosphatidylethanolamine and phosphatidylserine. DHA is liberated from the phospholipids by the action of calcium-independent phospholipase $A_2$ ($iPLA_2$). It then undergoes one of the following fates (McNamara and Carlson 2006):

1. Metabolism to several bioactive molecules, including the anti-inflammatory docosanoids
2. Degradation by β- oxidation or peroxidation
3. Reacylation into membrane phospholipids by acyltransferases
4. DHA can itself act as a second messenger and modulate synaptic signal transduction pathways.

## 1.30 Eicosapentaenoic acid (EPA)

EPA [5,8,11,14,17-*cis*-eicosapentaenoic acid, or, 20:5(n-3)] is one of the important long chain PUFA, the metabolites of which are predominantly associated with an anti-inflammatory effect.

EPA decreases the conversion of AA to $TXA_2$ in platelets and to $LTB_4$ in neutrophils, leading to a reduction in platelet aggregation and the inflammatory response, respectively. EPA may also compete with AA in the BMEC to form prostaglandins of the 'trienoic' series, which have an anti-inflammatory effect (for example, $PGE_3$) rather than metabolites of the 'dienoic' series, which are predominantly pro-inflammatory (for example, $PGE_2$). This could explain the anti-inflammatory effect of EPA and its role in protection against cardiovascular disease (Yerram at al. 1989).

### 1.31 Anti-inflammatory metabolites of EPA and DHA

EPA and DHA serve as precursors to a newly described family of biomolecules called specialized pro-resolving mediators (SPM) that include resolvins, protectins and maresins. The SPM are derived from EPA and DHA during the resolution phase of inflammation via transcellular interactions among inflammatory cells, tissue matrix and vasculature. The enzymes responsible for their formation are the cyclooxygenases and lipoxygenases (Bannenberg and Serhan 2010). EPA gives rise to resolvins (Rv) of the 'E' series, while DHA gives rise

to resolvins of the ‘D’ series, designated RvD1, 2, 3, 4 (Serhan 2005). Administration of aspirin interacts with the pathways of resolvin synthesis and leads to formation of the R-epimers denoted as “aspirin-triggered-RvDs” (Schwab and Serhan 2006). The resolvins act via specific receptors designated ResoDR1 and ResoER1 (Serhan *et al*. 2004).

**Table 1.1: Comparison of properties of the three prostaglandin synthases**.

| | **mPGES-1** | **mPGES-2** | **cPGES** |
|---|---|---|---|
| Chromosomal location | 9q34.4 | 9q33-q34 | 12q13.13 |
| mRNA size | 14.8 kb | 2 kb | 1.9 kb |
| Transcriptional regulation | Inducible | Constitutive | Constitutive |
| Molecular mass | 15-16 kDa | 33 kDa | 26 kDa |
| COX preference | Uses $PGH_2$ derived from COX-2 | Uses $PGH_2$ derived from both COX-1 and COX-2 | Uses $PGH_2$ derived from COX-1 |
| Structural properties | MAPEG family | Thioredoxin homology domain | Hsp 90 cochaperone p23 |
| Subcellular localization | Nuclear membrane | Golgi, cytosol | Cytosol |
| Tissue distribution | Prostate, testis, placenta, mammary gland. Can be induced in the brain in response to stimuli like LPS. | Brain, heart, skeletal muscle, kidney, liver | Ubiquitous |

(Samuelsson 2007; Park 2006; Kudo and Murakami 2005; Helliwell *et al*. 2003)

**Table 1.2: Comparison of properties of the four EP receptor subtypes.**

| | EP1 | EP2 | EP3 | EP4 |
|---|---|---|---|---|
| Chromosomal location | 19p13.1 | 14q22 | 1p31.2 | 5p13.1 |
| Cellular distribution | Fibroblasts | Smooth muscle | Variable | Smooth muscle |
| Second messenger | Ca2+/IP3 ($G_q$) | Increased cAMP ($G_s$) | Decreased cAMP ($G_i$) | Increased cAMP ($G_s$) |
| Agonists | ONO-DI-004<br>17-phenyl $PGE_2$ | ONO-AE1-259<br>CAY-10399<br>Butaprost-FA<br>CP-533536<br>19(R) –OH $PGE_2$ | ONO-AE-248<br>SC-46275<br>Sulprostone<br>MB-28767 | ONO-AE1-329<br>Tetrazolo-$PGE_1$<br>$PGE_2$ |
| Antagonists | SC-51089<br>SC-51322<br>ONO-8711 | PF-04418948<br>AH-6809 | DG-041<br>L-826266 | CJ-023423<br>CJ-042794<br>BGC-20-1531 |
| Kd for $PGE_2$ | >10 nM | >10 nM | <1 nM | <1 nM |
| Physiological actions | “Constrictor” receptor. Smooth muscle contraction in gastrointestinal, respiratory tracts, vas deferens, myometrium, iris sphincter. Regulates neurotransmitter release. | “Relaxant” receptor. | “Inhibitory” receptor. Regulation of NO synthesis in developing brain. At least eight splice variants known. | “Relaxant” receptor. |

(Negishi *et al*. 1995; Dumont *et al*. 1998; Varma *et al*. 1994; Bos *et al*. 2004; Nakao *et al*. 2007 Jones *et al*. 2009; Hata and Breyer 2004; Birrell and Nials 2011; Forselles *et al*. 2011)

**Table 1.3: Comparison of properties of the two COX isoforms.**

| | **COX-1** | **COX-2** |
|---|---|---|
| Expression | Constitutive | Inducible |
| Cellular functions | Housekeeping functions | Inflammatory functions |
| Tissue expression | Expressed in nearly all normal tissues | Not normally expressed, but can be induced in many organs in response to various stimuli |
| Chromosomal location | 9q32-q33.3 | 1q25.2-q25.3 |
| Inhibitors | No specific inhibitors | Specifically inhibited by the 'coxibs' (eg. Celecoxib) |
| mRNA size | 3 kb | 4-4.5 kb |
| Molecular mass | 72 kDa | 72 kDa |
| Km for arachidonic acid | ~ 5 µm | ~ 5 µm |
| Subcellular localization | ER, nuclear membrane | Nuclear membrane > ER |

(Park 2006; Dubois 1998; Turini and Dubois 2002)

**Table 1.4: Characteristics of the four leukotriene receptor subtypes.**

| | $BLT_1$ | $BLT_2$ | $CysLT_1$ | $CysLT_2$ |
|---|---|---|---|---|
| Ligands | $LTB_4$<br>20-OH-$LTB_4$<br>12-HETE | $LTB_4$<br>12-HETE<br>15-HETE | $LTC_4$<br>$LTD_4$ | $LTC_4$<br>$LTD_4$<br>$LTE_4$ |
| Antagonists | U75302<br>CP-105,696<br>ONO-4057 | LY-255,283<br>ONO-4057 | Montelukast<br>Zafirlukast<br>Pranlukast<br>BAYu9773 | BAYu9773 (partial)<br>CysLT2cpd |
| Chromosomal location | 14q11.2-q12 | 14q11.2-q12 | Xq13-21 | 13q14 |
| Amino acid residues | 352 | 389 | 337 | 346 |
| Tissue distribution | Leukocytes, spleen, thymus, lung | Leukocytes, spleen, liver, ovary | Leukocytes, spleen, thymus, lung, small intestine, prostate | Leukocytes, spleen, heart, placenta, kidney, ovary, brain |

(Back *et al*. 2011; Izumi *et al*. 2002)

# 2. OBJECTIVES OF THE STUDY

1. To study the transport of AA across primary cultured HBMEC monolayers as an in vitro model of the BBB.
2. To study the biological effects of incubating the HBMEC with AA, EPA and DHA.
3. To study the gene expression profile in HBMEC upon exposure to AA.

# 3. MATERIALS AND METHODS

## 3.1 Materials

[$^3$H] Arachidonic acid was obtained from Amersham Pharmacia Biotech (Baie d'Urfe, QC, Canada). Primary human brain microvascular endothelial cells (HBMEC) extracted and cultured from normal human brain cortical vessels and the cell culture medium (CSC Complete Classic Medium with Serum and CultureBoost) and reagents for growing and passaging these cells were obtained from Cell Systems Corporation (Kirkland, WA, USA). Opti-MEM reduced serum medium for transfection studies was obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Transwell® plates with polycarbonate inserts (0.4 μm; 24 mm diameter) were obtained from Corning Life Sciences (Lowell, MA, USA). Ecolite scintillant was obtained from ICN Biochemicals (Montreal, QC, Canada). Fluorescein dextran was obtained from Invitrogen (Burlington, ON, Canada). Prostaglandin $E_2$ and bovine serum albumin (BSA) was obtained from Sigma Chemical Company (St. Louis, MO, USA). RNeasy® Plus Mini Kit for RNA extraction was obtained from Qiagen (Cambridge, MA, USA). Primers for PCR were designed with the OligoPerfect™ Designer primer design tool (Invitrogen) and obtained from Invitrogen. FlexiTube siRNA for FATP-1 and CD36 were obtained from Qiagen. Lipofectamine™ RNAiMAX reagent was obtained from Invitrogen (Life

Technologies). All other biochemicals and drugs were certified ACS grade and obtained from either Fisher Scientific or Sigma Chemical Company. All cell culture flasks and dishes were obtained from Corning, Inc. (Corning, NY, USA).

### 3.2 Culturing of HBMEC

Frozen HBMEC were rapidly thawed in a 37$^{o}$C water bath and plated in a T75 flask and grown until 80-90% confluent. They were further passaged in a 1:3 split ratio into additional T75 flasks. The polycarbonate inserts of Transwell® plates to be used for permeability studies were first coated with Attachment factor™, a collagen- based extracellular matrix product, provided by the same supplier. HBMEC were plated on these inserts (70,000 cells per cm sq). Permeability studies were performed on confluent cells (typically after 3-4 days of culturing). The cells were maintained in CS-C complete medium that, in addition to a basal medium, contained 10% fetal bovine serum supplemented with ciprofloxacin and 50 μg/ml of CS-C growth factor (bovine Growth Factor and porcine heparin). Cells were incubated in a humidified incubator at 37$^{o}$C in 5% $CO_2$. Culture medium was replaced every 2 days until the cells were 50% confluent and then every day until the cells were 100% confluent. Cells used in the current study were passage 4 or lower.

### 3.3 Permeability studies

Confluent HBMEC monolayers were incubated apically with assay buffer #2 (122 mM sodium chloride, 2.9 mM potassium chloride, 1.9 mM calcium chloride, 2.5 mM magnesium sulfate, 25 mM sodium bicarbonate, 10 mM HEPES, 10 mM glucose and 0.4 mM dipotassium phosphate, pH 7.4) that contained 0.1 mM BSA, 0.1 mM arachidonic acid, [$^3$H] – arachidonic acid (2 μCi per dish) and 10 μM each of indomethacin, NS-398 and celecoxib. The basolateral medium consisted of only assay buffer #2 with 0.1 mM BSA. Incubation was carried out for up to 30 minutes. A 50 μl sample of basolateral medium was collected at time points of 0, 2, 5, 10, 20 and 30 minutes for measurement of radioactivity. The same volume of basolateral medium was added to the basolateral chamber to replenish the medium.

To confirm the radioactivity findings, fluorescein isothiocyanate-labeled dextran (FDX) at 1 μM concentration was used as a paracellular diffusion marker. A 20 μl aliquot of basolateral medium was sampled at the same time points after diluting with 200 μl of assay buffer. Fluorescence was determined using a Biotek Synergy HT plate reader. Fluorescence was detected at Ex (λ) 485 nm and Em (λ) 528 nm.

## 3.4 siRNA Transfection Studies

Transient transfection of HBMEC was done using the protocol described for HUVEC (Human umbilical vein endothelial cells) using Lipofectamine™ RNAiMAX reagent. Cells were seeded at a density of approximately 100,000 cells per well in six-well inserts and grown until 80-90% confluent (usually 24-48 hours). Prior to transfection, the cell culture medium was aspirated and the cells were incubated in Opti-MEM. The FATP-1 siRNA target sequence was 5'-ACAGTCGTCCTCCGCAAGAAA-3'. The CD36 siRNA target sequence was 5'-CAGGTGCTTAACACTAATTCA-3'. Complexes of 20 nM of FATP-1 or CD36 siRNA were formed by mixing the siRNA with Lipofectamine RNAiMAX reagent in Opti-MEM and incubating the mixture at room temperature for 20 minutes. The mixture was then added drop by drop to the cells in the Transwell® inserts while gently rocking the plate. Cells were incubated with these complexes at 37°C for 24 hours. Total RNA was isolated using the Qiagen RNeasy Plus Mini Kit to monitor gene silencing and the efficiency of transfection.

The transfected inserts were used for permeability and AA transport studies as previously described.

## 3.5 Prostaglandin $E_2$ studies

Confluent HBMEC monolayers were incubated apically with assay

buffer #2 (122 mM sodium chloride, 2.9 mM potassium chloride, 1.9 mM calcium chloride, 2.5 mM magnesium sulfate, 25 mM sodium bicarbonate, 10 mM HEPES, 10 mM glucose and 0.4 mM dipotassium phosphate, pH 7.4). The assay buffer contained 0.1 mM BSA, 0.1 mM arachidonic acid, [$^3$H] – arachidonic acid (2 μCi per dish) and serial doubling dilutions of prostaglandin $E_2$ (100, 50, 25 and 12.5 ng/ml). To some of the inserts with $PGE_2$, 10 μM of indomethacin or NS-398 was added. The basolateral medium consisted of only assay buffer #2 with 0.1 mM BSA. Incubation was carried out for up to 30 minutes. A 50 μl sample of basolateral medium was collected at time points of 0, 2, 5, 10, 20 and 30 minutes for measurement of radioactivity. The same volume of basolateral medium was added to the basolateral chamber to replenish the medium.

Similar permeability studies were performed using a 10-fold lower concentration of AA (10 μM) with equimolar amounts of BSA and 100 ng/ml of $PGE_2$ with or without 10 μM of indomethacin or NS-398. Fluorescein isothiocyanate-labeled dextran (FDX) at 1 μM concentration was used as a paracellular diffusion marker. A 20 μl aliquot of basolateral medium was sampled at the same time points after diluting with 200 μl of assay buffer. Fluorescence was determined using a Biotek Synergy HT plate reader. Fluorescence was detected at Ex (λ) 485 nm and Em (λ) 528 nm.

### 3.6 Gene Expression Studies and real-time PCR analysis

HBMEC were grown to confluence in 60 mm cell culture dishes. The cells were incubated for up to 30 minutes with 0.1 mM AA in plain cell culture medium (CSC) without added antibiotics or growth factors. Controls contained plain cell culture medium alone. At the end of each incubation period, cells were harvested and total RNA extracted and isolated using the RNeasy® Plus Mini kit (Qiagen). Measurement of gene expression by quantitative analysis was carried out using a Mastercycler ep *Realplex* system (Eppendorf). Human primers were designed by the OligoPerfect™ Designer software and synthesized by Invitrogen. Quantitative real-time RT PCR was carried out for analysis of gene expression using Quantitect Probe RT PCR SYBR Green kit (Qiagen) and qScript™ One-Step SYBR® Green qRT-PCR kit (Quanta Biosciences). PCR amplification of the housekeeping gene 18S rRNA was carried out as a control for sample loading and to allow normalization among samples. The samples were heated in the thermal cycler for 15 minutes at 95$^{o}$C for reverse transcription, followed by 40 cycles of denaturation at 95$^{o}$C for 15 seconds, annealing at 60$^{o}$C for 30 seconds and extension at 72$^{o}$C for 30 seconds. Relative gene expression was determined as previously described with 18S rRNA as control. $\Delta\Delta C_T$ values were calculated by subtracting the $\Delta C_T$ of the control (baseline conditions) from the $\Delta C_T$ of the sample

(experimental conditions). The 18S rRNA control was normalized to 1.0 and each target gene was then normalized and represented as fold-change compared to 18S rRNA (Saini-Chohan *et al.* 2011). Primers used for the analysis are shown in **Table 3.1**.

### 3.7 Mass Spectrometry Studies

(Procedure for mass spectrometry provided by Dr. Harold Aukema, Human Nutritional Sciences, University of Manitoba. Mass spectrometry performed by Mr. Joy Gauthier in the laboratory of Dr. Harold Aukema).

HBMEC were grown to confluence in 60 mm cell culture dishes. The cells were incubated for 30 minutes with plain cell culture medium, devoid of growth supplements or antibiotics, to which 0.1 mM AA was added. The culture medium was harvested at time points of 2, 5, 10, 20 and 30 minutes. An antioxidant cocktail was added to the aliquot in a concentration of 3.33 µl per ml of medium to stabilize the metabolites. The antioxidant cocktail consisted of 0.2 mg/ml of BHT, 0.2 mg/ml of EDTA, 2 mg/ml of TPP and 2 mg/ml of indomethacin in a 2:1:1 proportion of methanol : ethanol : water as solvent.

An internal standard mix consisting of 7.5 – 25 ng of deuterated standards (see below) was added to the aliquots, acidified to pH < 3.0 with 1N HCl, and applied to Strata-X SPE columns (Phenomenex, Torrance, CA) pre-conditioned with methanol and water (pH 3). After loading, columns

were washed in 10% methanol in water (pH 3) and samples eluted with 100% methanol. Liquid chromatography tandem mass spectrometry (LC/MS/MS) was performed as described (Deems *et al.* 2007). Dried down samples were re-suspended in water/acetonitrile/formic acid (70:30:0.02, v/v/v, solvent A) and eicosanoids separated by reverse-phase HPLC using a C18 column (Luna, 250 x 2.0mm, Phenomenex, Torrance, CA) at a flow rate of 300 µL/min. The column was equilibrated in solvent A and samples were eluted with a linear gradient from 0 to 20 % solvent B (acetonitrile/isopropyl alcohol, 50:50; v/v) for 11 min, then increased to 100% by 13 min and held until 16 min, then dropped to 0% by 16 min and held until 19 min. The HPLC was coupled to a triple quadrupole tandem mass spectrometer (API 2000) with electrospray ionization source (Applied Biosystems, Concord, ON, Canada). Eicosanoid analysis was via multiple-reaction monitoring (MRM) in negative-ionization mode. Mass transitions of deuterated standards and eicosanoids were as follows: 15-HETE-$d_8$ (*m/z* 327→226) for 5-HETE (*m/z* 319→115), 8-HETE (*m/z* 319→155), 9-HETE (*m/z* 319→151), 11-HETE (*m/z* 319→167), 12-HETE (*m/z* 319→179) and 15-HETE (*m/z* 319→219),; $LtB_4$-$d_4$ (*m/z* 339→197) for $LtB_4$ (*m/z* 335→195) and 5,6-$LxA_4$ (*m/z* 351→115); $TxB_2$-$d_4$ (*m/z* 373→173) for $TxB_2$ (*m/z* 369→169); $PGF_{2\alpha}$-$d_4$ (*m/z* 357→197) for $PGF_{2\alpha}$ (*m/z* 353→193); $PGE_2$-$d_4$ (*m/z* 355→275) for $PGE_2$ (*m/z* 351→271); $PGD_2$-$d_4$ (*m/z* 355→193) for $PGD_2$ (*m/z* 351→189). Quantification of

eicosanoids was determined as described (Deems *et al.* 2007).

### 3.8 Statistics

All data were expressed as mean SE. Comparisons among the different groups were evaluated by one-way ANOVA followed by the Dunnett test for multiple comparisons of the means. The tests were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, CA. *p* values less than 0.05 were considered statistically significant.

**Table 3.1 : Primers used for real-time PCR analysis**

| Primer | Forward 5'-3' | Reverse 5'-3' |
|---|---|---|
| 18S | AAACGGCTACCACATCCAAG | CCTCCAATGGATCCTCGTTA |
| EP1 | TTGTCGGTATCATGGTGGTG | ATGTACACCCAAGGGTCCAG |
| EP2 | CCACCTCATTCTCCTGGCTA | TTCCTTTCGGGAAGAGGTTT |
| EP3 | AGCTTATGGGGATCATGTGC | TTTCTGCTTCTCCGTGTGTG |
| EP4 | GACCTGTTGGGCACTTTGTT | AGGTAGCGCTCGACACTCAT |
| mPGES | GGAACGACATGGAGACCATC | GGAAGACCAGGAAGTGCATC |
| cPGES | AAGGAGAATCTGGCCAGTCA | ATCCTCATCACCACCCATGT |
| COX-1 | CTTTTCCCTCAAGGGTCTCC | AGGGACAGGTCTTGGTGTTG |
| COX-2 | TGAGCATCTACGGTTTGCTG | TGCTTGTCTGGAACAACTGC |
| 5-LOX | TCATCGTGGACTTTGAGCTG | GACAATCTTGTTGGCCAGGT |
| 12-LOX | CTCTGCCATTTCCTCACCAT | GCCATCGTCACATCTTCCTT |
| $PGD_2R$ | CTACGCTCAGAACCGGAGTC | GTGCCGTCGGTAGAAGAAAG |
| $LxA_4R$ | ACTGCTGTGGTGGCTTCTTT | ACGTAAAGCATGGGGTTGAG |
| CysLTR | TGACCGCTGCCTTTTTAGTC | ATGCAGCCAGAGACAAGGTT |
| PGT | GTGGTGAACCAGGAGGAAAA | AGGAGTGGTCAATGGTGAGG |
| MRP4 | CCATCTGTGCCATGTTTGTC | ACTGAAACATCCCCATGAGC |
| PGDH | TGCTTCAAAGCATGGCATAG | AACAAAGCCTGGACAAATGG |
| Claudin-1 | CCGTTGGCATGAAGTGTATG | AGCCAGACCTGCAAGAAGAA |
| Claudin-3 | CACGCGAGAAGAAGTACACG | GTAGTCCTTGCGGTCGTAGC |

| | | |
|---|---|---|
| Claudin-5 | TGTCGCAGAAGTACGAGCTG | GTACTTCACGGGGAAGCTGA |
| Claudin-12 | TGTCCTCCAGTTTGCCCTAC | CAGACACTTGGCCAGTTTGA |
| Occludin | TTTGTGGGACAAGGAACACA | TCATTCACTTTGCCATTGGA |
| JAM-1 | TCAAGGTCAAGCTCATCGTG | GGGGAACCATCTTGTTCTGA |
| JAM-3 | GACTTCTTCCTGCTGCTGCT | ATCCTGGGGTCACTTGTCTG |
| ZO-1 | GTCTGCCATTACACGGTCCT | GGCTTAAATCCAGGGGAGTC |
| ZO-2 | GCTTGGGAGTCAGATCTTCG | CTGGCTGTCTCTCAACACCA |
| ZO-3 | GTGCAGATGAAGCCTGTGAA | AGGCCCGAATCTGTAATGTG |
| CLS-1 | AATGACGAGAATTGGCTTGG | TCTTTGATTGGCCCAGTTTC |
| GLUT-1 | TCACTGTGCTCCTGGTTCTG | CCTCGGGTGTCTTGTCACTT |
| PPAR-γ | GCTGGCCTCCTTGATGAATA | TTGGGCTCCATAAAGTCACC |
| MMP-2 | ATGACAGCTGCACCACTGAG | AGTTCCCACCAACAGTGGAC |
| MMP-3 | TGCTTTGTCCTTTGATGCTG | GGAAGAGATGGCCAAAATGA |
| MMP-9 | TTGACAGCGACAAGAAGTGG | GCCATTCACGTCGTCCTTAT |

# 4. RESULTS

To study the transport of arachidonic acid (AA) across the HBMEC monolayer, the cells were grown as a confluent monolayer on polycarbonate Transwell® membrane inserts. The HBMEC were incubated apically with 0.1 mM AA bound to bovine serum albumin (BSA) in a 1:1 molar ratio. To study the paracellular flux, FDX was added as the marker molecule. It was observed that there was an immediate increase in the paracellular flux of FDX across the HBMEC monolayer when incubated with AA as compared to the control monolayer without AA. The FDX flux at 2 minutes was 2.84(±0.16)% in the control insert versus 9.25(±0.3)% in the inserts with AA. Similarly, the flux was 3.58(±0.21)% versus 9.91(±0.32)% at 5 minutes and 4.75(±0.32)% versus 10.18(±0.2)% at 10 minutes. The difference gradually leveled off by 30 minutes of incubation. The data are shown in **Figure 4.1**.

Permeability studies were then carried out on HBMEC monolayer incubated with AA in the presence of specific and non specific inhibitors of cyclooxygenase (COX). Primary HBMEC at passage 3 were grown to confluence on polycarbonate Transwell® inserts. The cells were incubated apically with 0.1 mM AA bound to albumin in a 1:1 molar ratio in the presence of indomethacin, a non specific COX

inhibitor and with celecoxib and NS-398, both specific inhibitors of COX-2. [$^3$H]AA was added as the tracer molecule and radioactivity incorporated in the basolateral medium was determined at time points of 2, 5, 10, 20 and 30 minutes. There was an increase in the radioactivity in the basolateral medium over 30 minutes of incubation. The presence of COX inhibitors did not significantly affect the passage of [$^3$H]AA from the apical to the basolateral side. The data are shown in **Figure 4.2**.

We next decided to study if the fatty acid transport proteins (FATP1, CD36) had a role to play in the transport of AA. Primary HBMEC at passage 3 were grown to confluence on polycarbonate Transwell® inserts. The genes for FATP1 and CD36 were individually knocked down using siRNA after the cells were 80-90% confluent on the inserts (after approximately 24 hours). The HBMEC were then incubated apically with 0.1 mM AA bound to BSA in a 1:1 molar ratio. [$^3$H]AA was added as the tracer molecule and the radioactivity incorporated in the basolateral medium was determined over 30 minutes of incubation at time points of 2, 5, 10, 20 and 30 minutes. No significant difference in the movement of [$^3$H]AA was observed after knocking down the genes for either FATP1 or CD36 as compared to the HBMEC monolayers with intact FATP1 and CD36. The data are shown in **Figure 4.3 (A,B)**.

As an extension to this study, some of the HBMEC monolayers with FATP1 and CD36 knocked-down were also incubated with the COX inhibitor drugs in addition to AA. Again, no significant difference was seen in the movement of tracer [$^{3}$H]AA from the apical to the basolateral side of the monolayer over 30 minutes of incubation. This data are shown in **Figure 4.3 (C,D).**

To further explore the role of fatty acid transporters in arachidonate transport, we decided to study the permeability of the HBMEC monolayer after incubating the cells with 200 µM phloretin, a non specific inhibitor of fatty acid uptake. Primary HBMEC at passage 3 were grown to confluency on polycarbonate Transwell® inserts. The HBMEC were incubated apically with 0.1 mM AA bound to BSA in a 1:1 molar ratio in the presence or absence of phloretin. FDX was used as the marker molecule to study the paracellular flux. No significant change was observed in the permeability of the HBMEC monolayer on incubating the cells with phloretin for up to 30 minutes as compared to control monolayers without phloretin. The FDX fluorescence was measured over time points of 2, 5, 10, 20 and 30 minutes. The data are shown in **Figure 4.4 (A)**.

As an extension of this experiment, some of the HBMEC monolayers were also incubated with the COX inhibitor drugs in addition to

phloretin. No significant change in permeability was seen when the cells were incubated with the COX inhibitors together with phloretin for up to 30 minutes. The data are shown in **Figure 4.4 (B)**.

Next we decided to study if addition of $PGE_2$ altered the permeability of the HBMEC monolayer. Primary HBMEC at passage 3 were grown as a confluent monolayer on polycarbonate Transwell® inserts. HBMEC were incubated with 0.1 mM AA bound to BSA in a 1:1 molar ratio in the presence or absence of 100 ng/ml of $PGE_2$ and the COX inhibitor drugs. [$^3$H]AA was added as the tracer molecule and radioactivity incorporated in the basolateral medium was measured at time points of 2, 5, 10, 20 and 30 minutes. As in our previous studies, the presence of COX inhibitors did not alter the movement of [$^3$H]AA from the apical to basolateral side. However, incubation with $PGE_2$ reduced the movement of [$^3$H]AA from the apical to the basolateral medium by 20% at 20 minutes and 45% at 30 minutes of incubation. The data are shown in **Figure 4.5**.

We next decided to examine if $PGE_2$ has a similar effect at lower concentrations. Primary HBMEC (P3) were grown as a confluent monolayer on polycarbonate Transwell® inserts. HBMEC were incubated with 0.1 mM AA bound to BSA in a 1:1 molar ratio in the

presence or absence of COX inhibitors and three different concentrations of $PGE_2$: 50 ng/ml, 25 ng/ml and 12.5 ng/ml. [$^3$H]AA was added in trace amounts (2 μCi per dish). As observed in previous experiments, the presence of COX inhibitors did not alter the movement of AA from the apical to basolateral side. However, at all the three concentrations studied, $PGE_2$ had a significant effect in decreasing the transport of AA into the basal chamber. This movement of AA decreased by 14-24% at 10 minutes, 23-30% at 20 minutes and 33% at 30 minutes. The results are shown in **Figure 4.6**.

We next decided to study if a 10-fold lower concentration of AA (10 μM) would have a similar effect on increasing the permeability of the HBMEC monolayer. Primary HBMEC (P3) were grown as a confluent monolayer on polycarbonate Transwell® inserts and incubated with 10 μM of AA bound to BSA in an equimolar ratio in the presence or absence of COX inhibitor drugs and 100 ng/ml of $PGE_2$. FDX was added as a marker to assess the paracellular passage of AA from the apical to the basolateral chamber. The results are shown in **Figure 4.7**.

Similar to previous studies with 100 μM of AA, increased permeability of the HBMEC monolayer was seen with 10 μM of AA. Also, this increase in permeability was significantly attenuated on incubating

the cells with 100 ng/ml of $PGE_2$. $PGE_2$ reduced the permeability of the HBMEC monolayer by 16% at 2 minutes, 26% at 5 minutes, 40% at 10 minutes, 52% at 20 minutes and 54% at 30 minutes.

It was next decided to study if incubation of HBMEC with arachidonic acid leads to a change in the production of various eicosanoids. Primary HBMEC at passage 3 were grown as a confluent monolayer in 60 mm culture dishes. The cells were incubated for up to 30 minutes with 0.1 mM arachidonic acid bound to BSA in a 1:1 molar ratio in plain cell culture medium devoid of antibiotics or additional growth factors (Culture Boost). The cell culture medium was harvested at time points of 2, 5, 10, 20 and 30 minutes. Mass spectrometry was performed for quantitation of the various eicosanoids produced. The data are shown in **Table 4.1** and represented graphically in **Figure 4.8 (A-L)**. For obtaining the baseline (control) readings of the eicosanoids profile, the HBMEC were incubated with only plain culture medium for 30 minutes, without the addition of arachidonic acid.

Incubation of the HBMEC with arachidonic acid led to an immediate, increased production of various eicosanoids at two minutes of incubation compared to the baseline concentrations. This increase persisted throughout the 30-minute incubation period for most of the metabolites. The metabolites whose concentration was markedly

increased from baseline were $PGE_2$, $PGD_2$, 5,6-$LxA_4$ and 5-HETE. On the other hand, the levels of $LTB_4$ decreased as compared to baseline levels.

It was next decided to study if incubation of HBMEC with arachidonic acid influences the transcription of genes involved in the production, action and metabolism of eicosanoids and those involved in the formation and maintenance of tight junctions between endothelial cells and hence, regulate permeability across the HBMEC monolayers. Primary HBMEC in passage 3 were grown to confluence in 60 mm culture dishes. The cells were incubated for up to 30 minutes with 0.1 mM AA bound to BSA in a 1:1 molar ratio in plain cell culture medium, devoid of antibiotics or growth factors. The cells were harvested at time points of 2, 5, 10, 20 and 30 minutes and their total RNA was extracted. Real-time PCR was performed for relative quantitation of select genes using 18S rRNA gene as the housekeeping gene. Of the several genes studied, incubation with AA led to the up regulation of genes for EP4 receptor, JAM-1 and claudin-3. Several genes were down regulated on incubation with AA including the EP1 receptor, EP3 receptor, $PGD_2$ receptor, $LxA_4$ receptor, cysteinyl leukotriene receptor, claudin-1, claudin-12, COX-1, prostaglandin transporter (PGT), prostaglandin dehydrogenase, 12-lipoxygenase, ZO-3, MMP-9 and

PPAR-γ. The expression of several other genes was unchanged. The data are shown in **Figure 4.9 (A-A6)**.

Next, we decided to study the flux of FDX across the HBMEC monolayers on incubation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Primary HBMEC at passage 3 were grown to confluence on polycarbonate Transwell® inserts. The cells were incubated with 0.1 mM EPA and DHA, separately, bound to BSA in a 1:1 molar ratio. FDX flux across the monolayer was measured for up to 30 minutes of incubation. There was no significant change in the FDX flux across the HBMEC monolayers incubated with either EPA or DHA as compared to the monolayers without added EPA or DHA. The data are shown in **Figure 4.10 (A,B)**.

We next decided to study if incubation of HBMEC with EPA or DHA influences the production of various metabolites of the long chain PUFA. Primary HBMEC at passage 3 were grown to confluence in 60 mm cell culture dishes. The cells were incubated for up to 30 minutes with 0.1 mM EPA or DHA bound to BSA in a 1:1 molar ratio in plain cell culture medium, devoid of antibiotics or added growth factors. The cell culture medium was harvested at time points of 2, 5, 10, 20 and 30 minutes. For obtaining a baseline (control) profile of EPA and DHA

metabolites, the HBMEC were incubated with only plain cell culture medium for 30 minutes, without the addition of EPA or DHA. Mass spectrometry was performed for quantitative analysis of the various metabolites produced.

Incubation with EPA led to a marked increase in the production of prostanoids of the '3' series, catalyzed by the COX pathway, namely, $TxB_3$, $PGE_3$ and $PGD_3$. Similarly, there was a marked increase in the production of $LxA_4$ and the various HEPE (hydroxy eicosapentaenoic acid) metabolites produced via the lipoxygenase pathway. The data are shown in **Table 4.3** and presented graphically in **Figure 4.11**.

Incubation with DHA led to a marked increase in the production of various HDoHE (hydroxy docosahexaenoic acid) metabolites via the lipoxygenase pathway. Notably, DHA incubation also led to an increase in the concentrations of maresin, resolvins and protectin, endogenous anti-inflammatory compounds produced specifically by DHA. The data are shown in **Table 4.4** and presented graphically in **Figure 4.12**.

**Figure 4.1: Incubation of HBMEC with AA increases FDX flux across HBMEC monolayers.**

HBMECs were incubated with 0.1 mM AA bound to albumin (1:1 molar ratio) and FDX. FDX incorporation into the basolateral medium determined as described in Materials and Methods. Data represents the mean of three experiments.

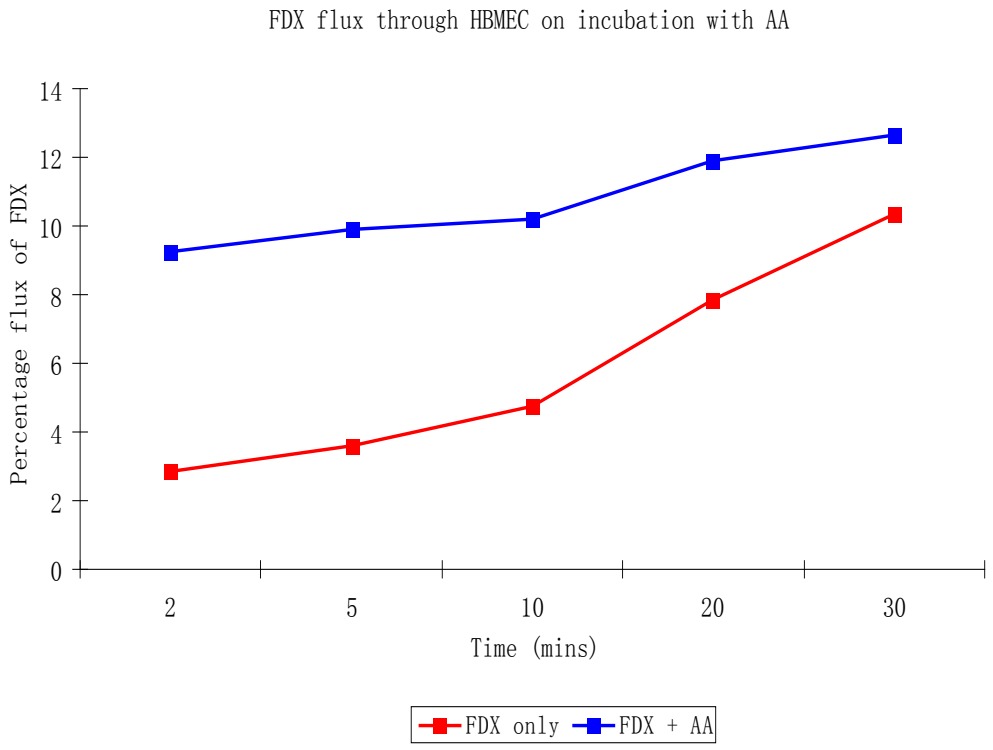

**Figure 4.2: Incubation with COX inhibitors does not alter the permeability of HBMEC monolayers.**

HBMEC were incubated apically with 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 min in the absence or presence of 10 μM indomethacin or NS-398 or celecoxib. [$^3$H]AA was added as the tracer in a dose of 2 μCi per well and radioactivity incorporated into the basolateral medium determined. Data represents the mean ± SE of three experiments.

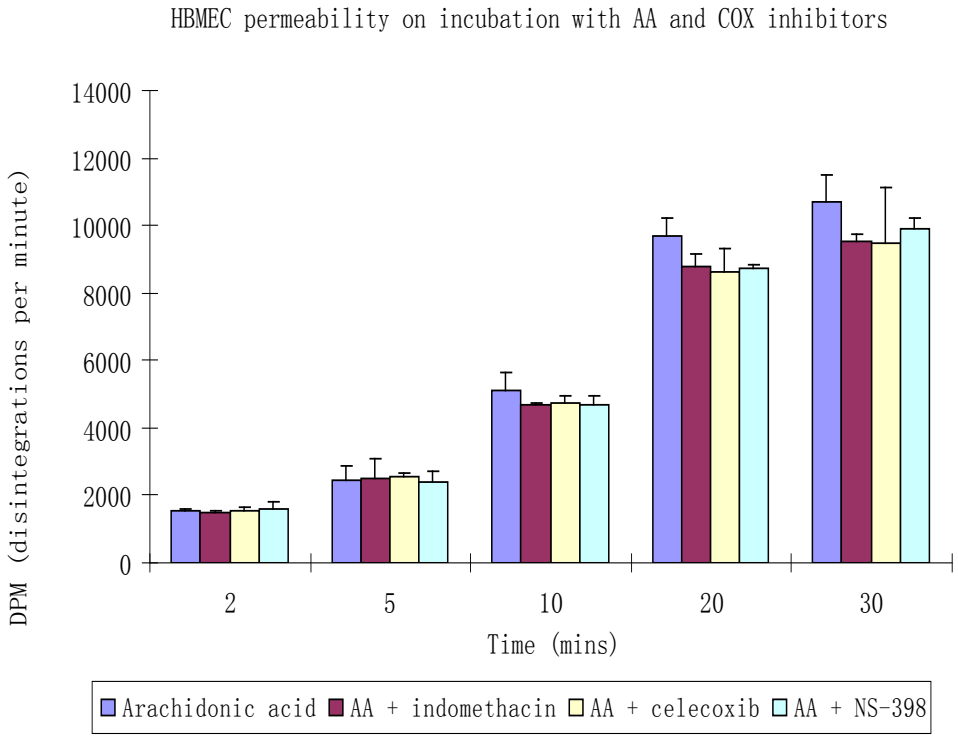

**Figure 4.3: Knockdown of FATP1 or CD36 does not alter the movement of [$^3$H]AA across HBMEC monolayers.**

HBMEC were transfected with FATP-1 (A) or CD36 siRNA (B) for 48 h as described in Materials and Methods. HBMEC were then incubated apically with 0.1 mM AA bound to albumin (1:1 molar ratio) with or without 10 μM COX inhibitor drugs (C and D) for up to 30 min. [$^3$H]AA was added as a tracer in a dose of 2 μCi per well and radioactivity incorporated into the basolateral medium determined as described in Materials and Methods. Data represents the mean ± SE of three experiments.

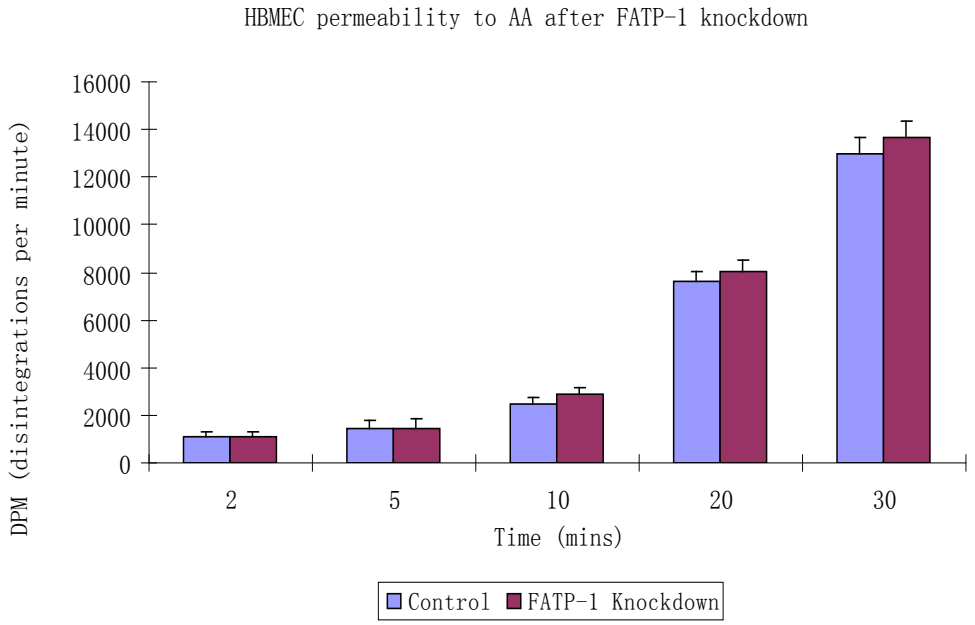


**Figure 4.3 (A)**

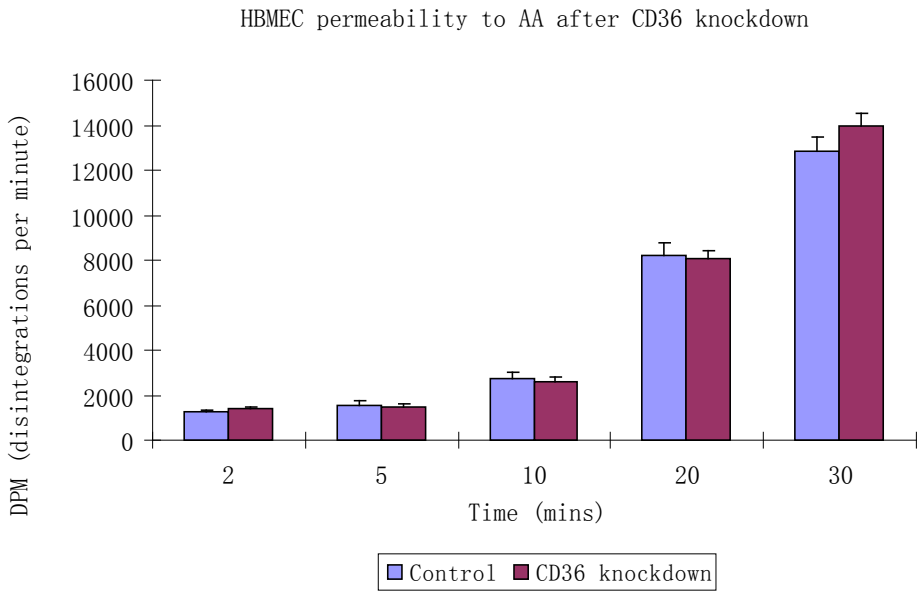
HBMEC permeability to AA after CD36 knockdown
DPM (disintegrations per minute)
16000
14000
12000
10000
8000
6000
4000
2000
0
2
5
10
20
30
Time (mins)
Control
CD36 knockdown

**Figure 4.3 (B).**

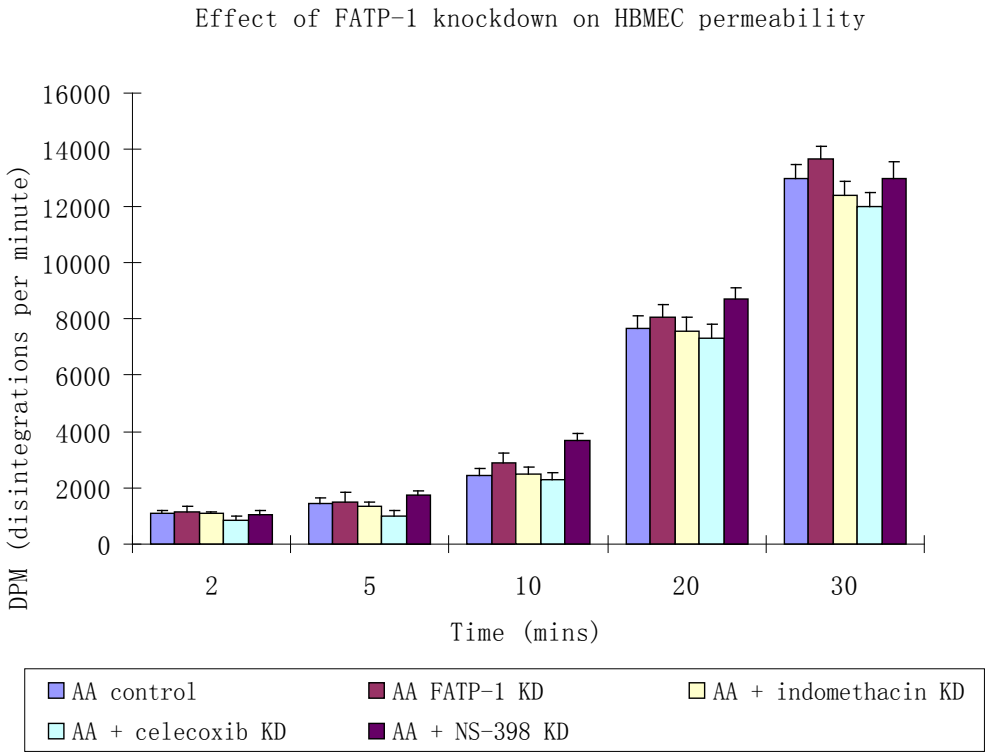
Effect of FATP-1 knockdown on HBMEC permeability
DPM (disintegrations per minute)
16000
14000
12000
10000
8000
6000
4000
2000
0
2
5
10
20
30
Time (mins)
AA control
AA FATP-1 KD
AA + indomethacin KD
AA + celecoxib KD
AA + NS-398 KD

**Figure 4.3 (C)**

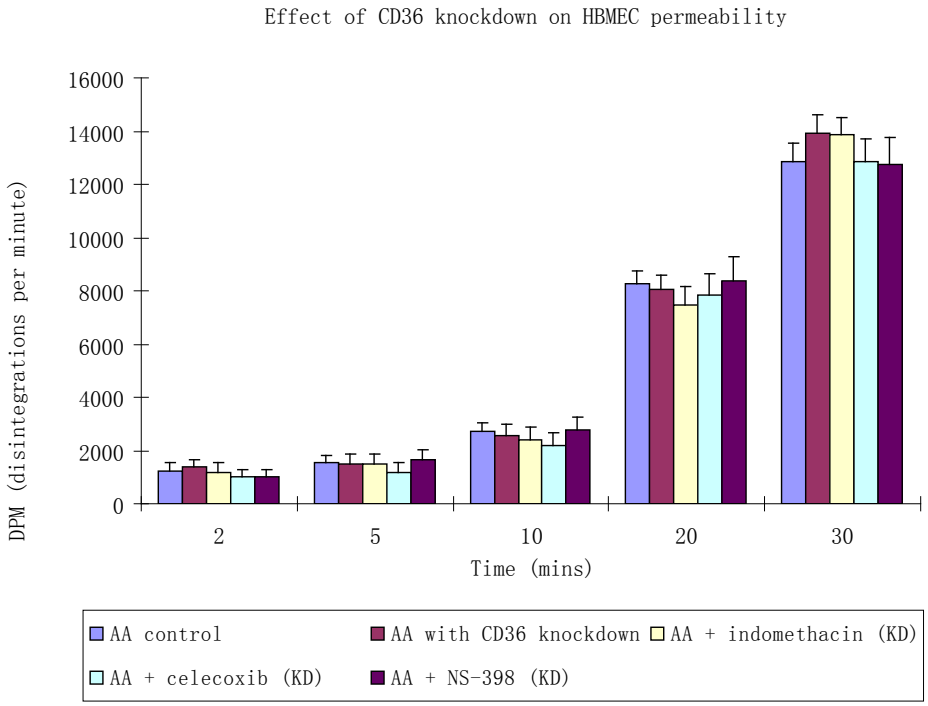
Effect of CD36 knockdown on HBMEC permeability
DPM (disintegrations per minute)
16000
14000
12000
10000
8000
6000
4000
2000
0
2
5
10
20
30
Time (mins)
AA control
AA with CD36 knockdown
AA + indomethacin (KD)
AA + celecoxib (KD)
AA + NS-398 (KD)

**Figure 4.3 (D)**

**Figure 4.4 (A, B): Blockade of fatty acid transport proteins with phloretin does not affect the permeability of HBMEC monolayers.**

HBMEC were incubated apically with 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 min with phloretin (A) in the absence or presence of 10 μM indomethacin or NS-398 (B) and the flux of 1 μM FDX across the HBMEC monolayers was determined. Data represents the mean ± SE of three experiments.

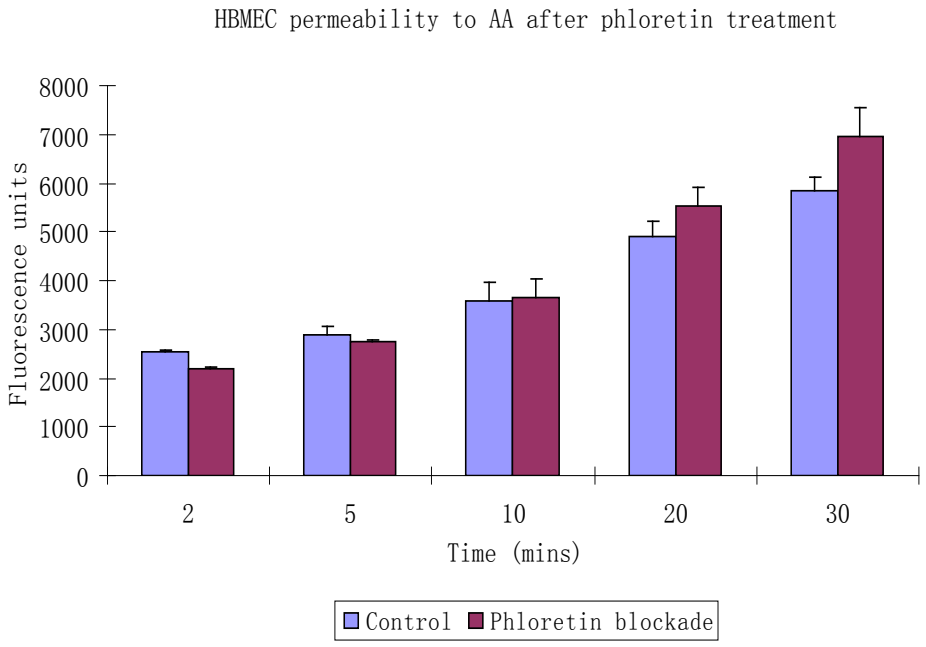


**Figure 4.4 (A)**

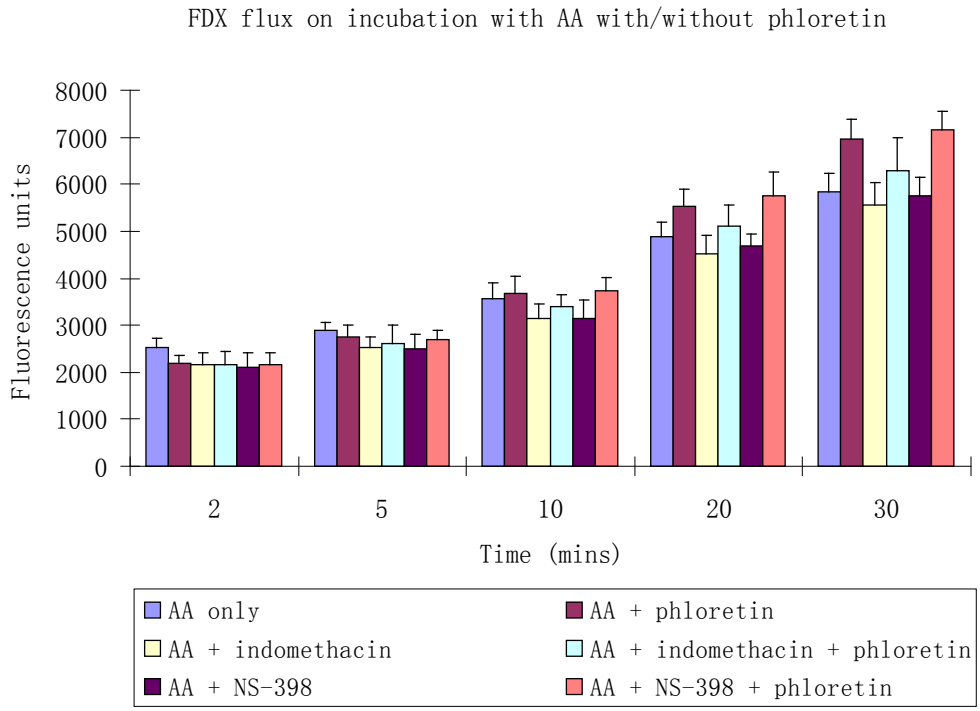
FDX flux on incubation with AA with/without phloretin
Fluorescence units
8000
7000
6000
5000
4000
3000
2000
1000
0
2
5
10
20
30
Time (mins)
AA only
AA + phloretin
AA + indomethacin
AA + indomethacin + phloretin
AA + NS-398
AA + NS-398 + phloretin

**Figure 4.4 (B)**

**Figure 4.5: $PGE_2$ attenuates the transport of [$^3$H]AA across the HBMEC monolayers.**

HBMEC were incubated apically with 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 min in the absence or presence of 10 μM indomethacin or NS-398 and in the presence or absence of 100 ng/ml of $PGE_2$. [$^3$H]AA was added as a tracer in a dose of 2 μCi per well and radioactivity incorporated into the basolateral medium determined. Data represents the mean ± SE of three experiments. *p<0.05, **p<0.01

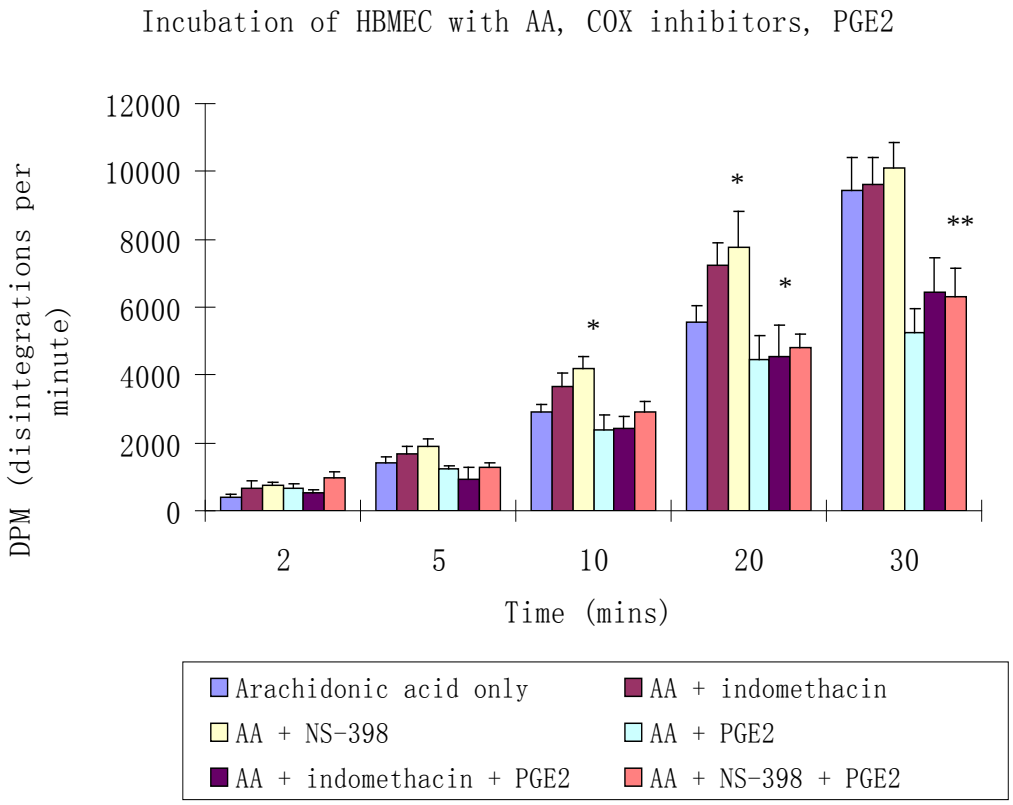

**Figure 4.6: $PGE_2$ attenuates the transport of [$^3$H]AA across the HBMEC monolayers even at lower concentrations.**

HBMEC were incubated apically with 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 min in the absence or presence of 10 μM indomethacin or NS-398 and in the presence or absence of 50, 25 or 12.5 ng/ml of $PGE_2$. [$^3$H]AA was added as a tracer in a dose of 2 μCi per well and radioactivity incorporated into the basolateral medium determined. Data represents the mean ± SE of three experiments. *p<0.05; ***p<0.001

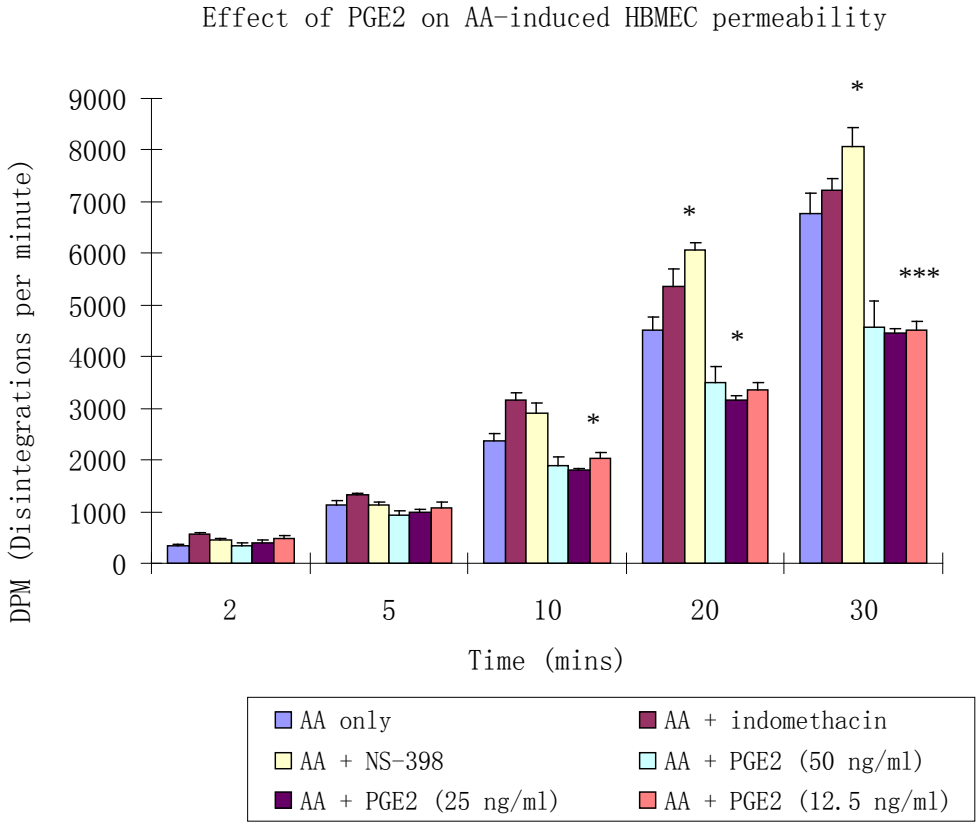

**Figure 4.7: $PGE_2$ attenuates the AA-mediated permeability of HBMEC monolayers.**

HBMECs were incubated with 10 μM AA bound to BSA (1:1 molar ratio) and 1 μM FDX in the absence or presence of the COX inhibitors indomethacin or NS-398 or with 100 ng/ml of $PGE_2$ and FDX flux into the basolateral medium determined as described in Materials and Methods. Data represents the mean ± SE of three experiments. ****p<0.0001.

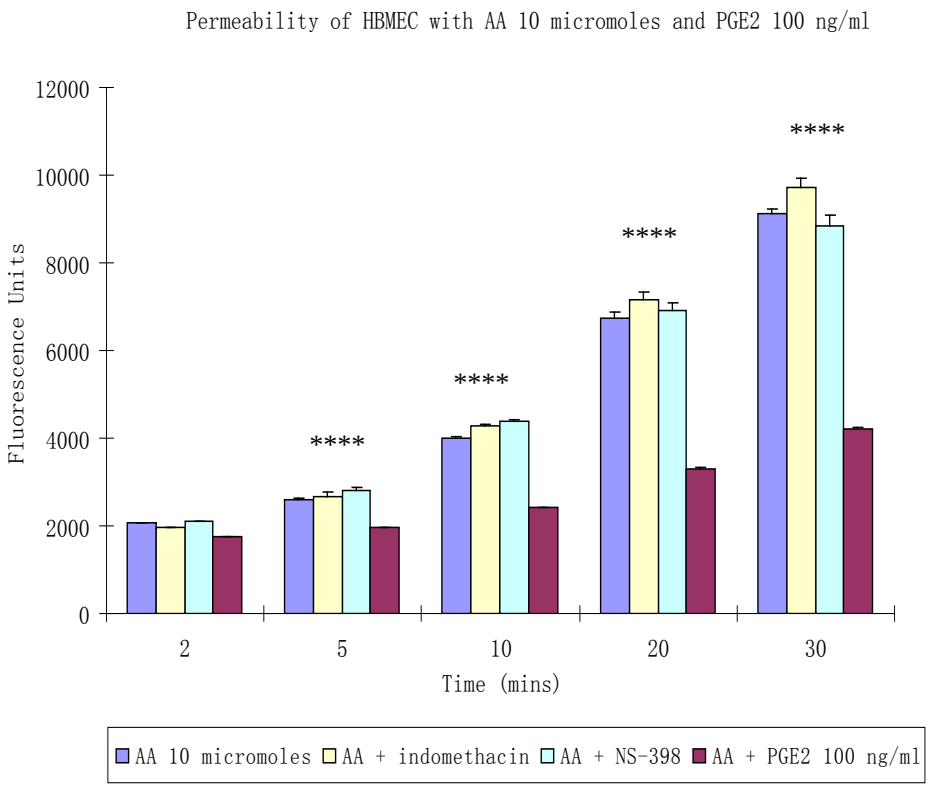

**Figure 4.8 (A-L): AA metabolites generated by HBMEC**

HBMECs were incubated in the absence or presence of 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 minutes and AA metabolites determined by mass spectrometry analysis as described in Materials and Methods.

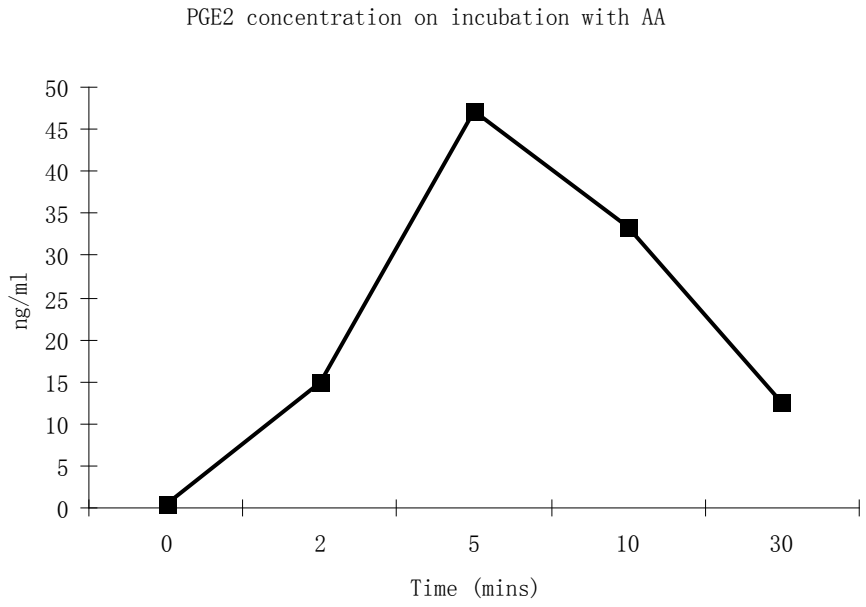


**Figure 4.8 (A)**

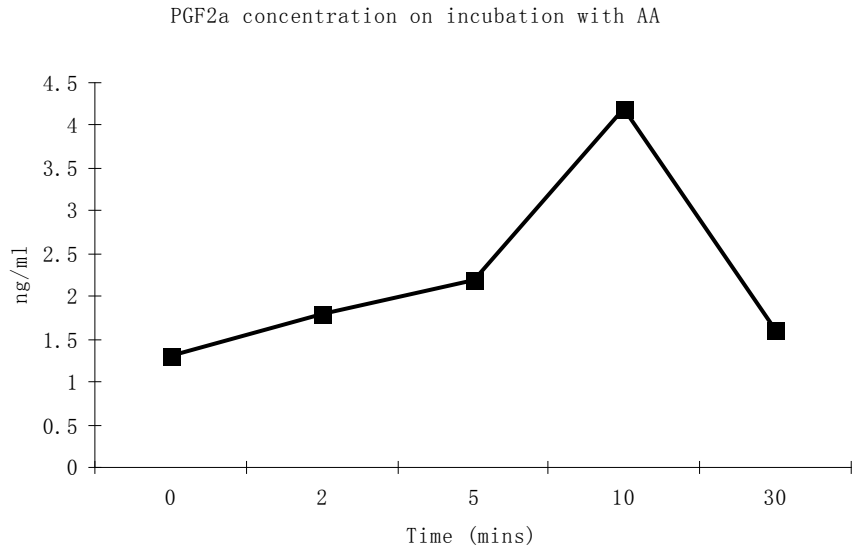


**Figure 4.8 (B)**

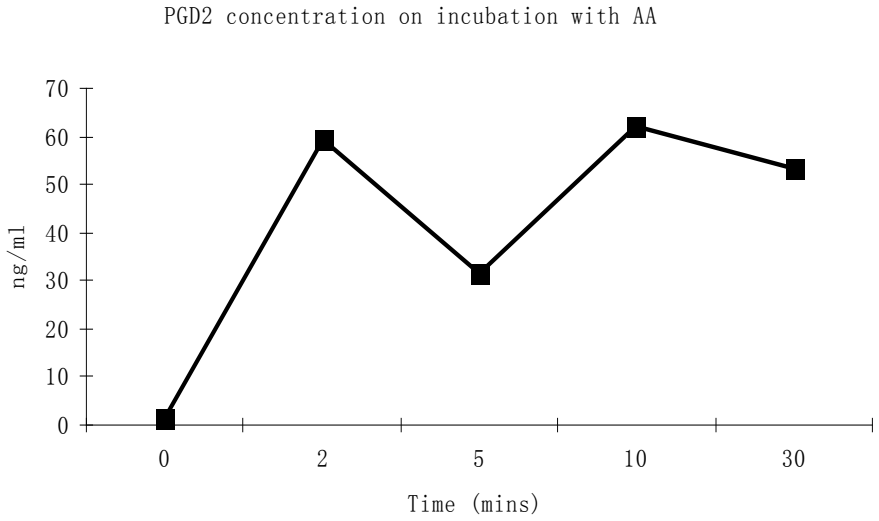


**Figure 4.8 (C)**

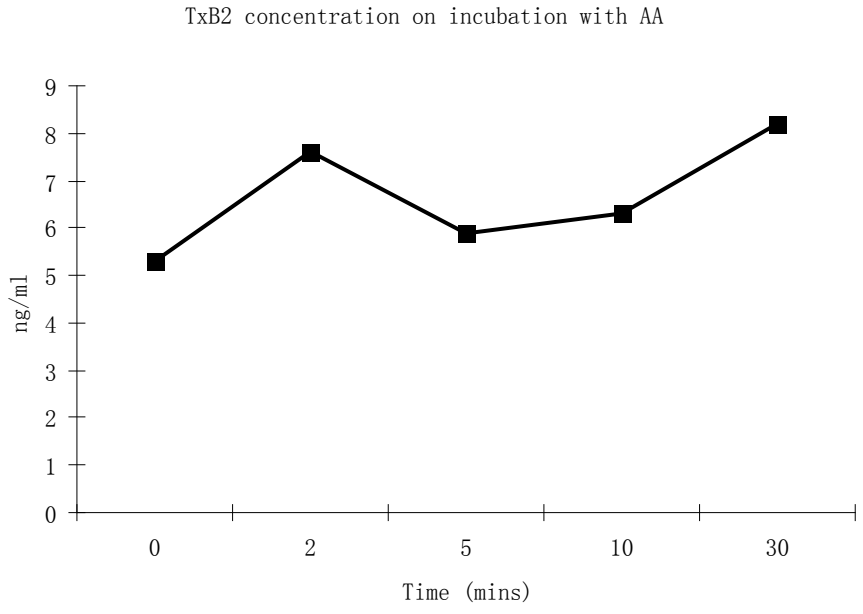


**Figure 4.8 (D)**

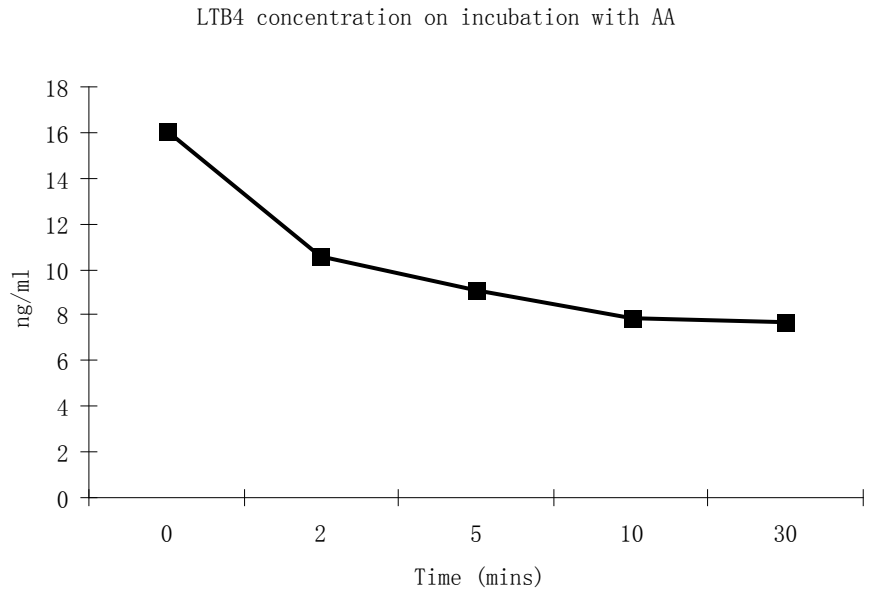


**Figure 4.8 (E)**

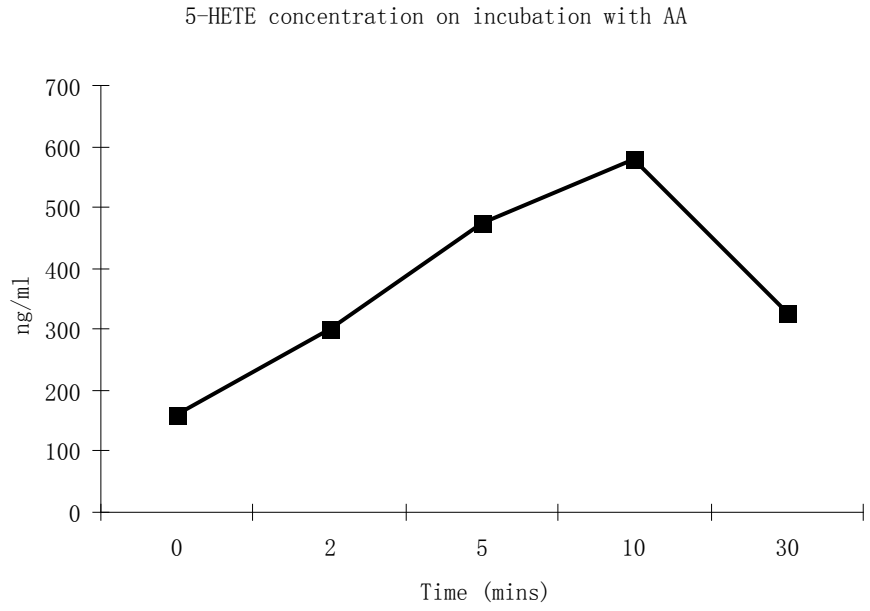


**Figure 4.8 (F)**

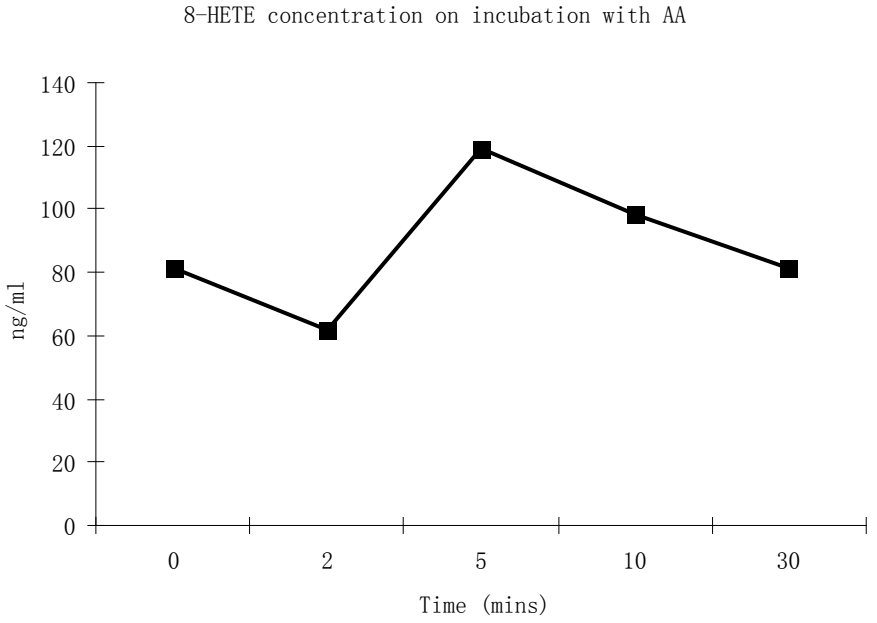


**Figure 4.8 (G)**

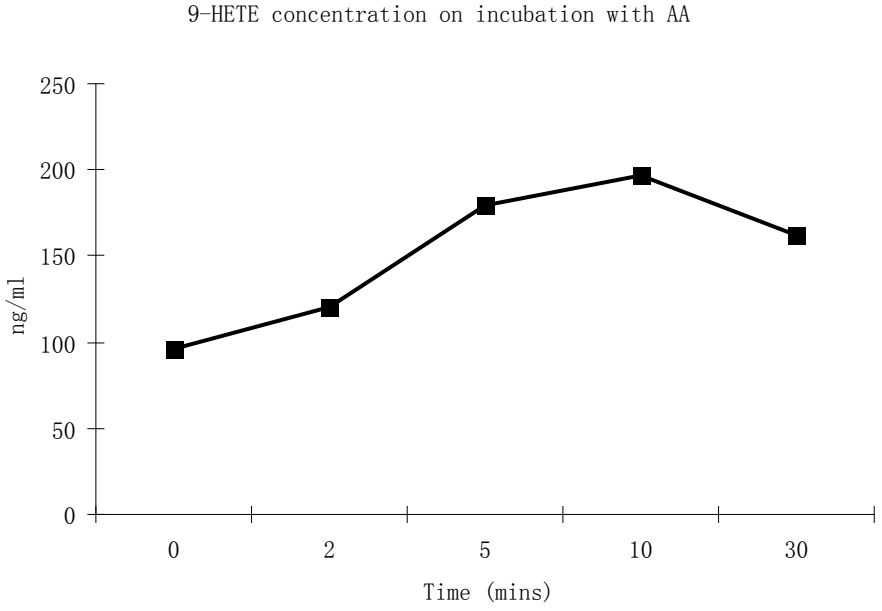


**Figure 4.8 (H)**

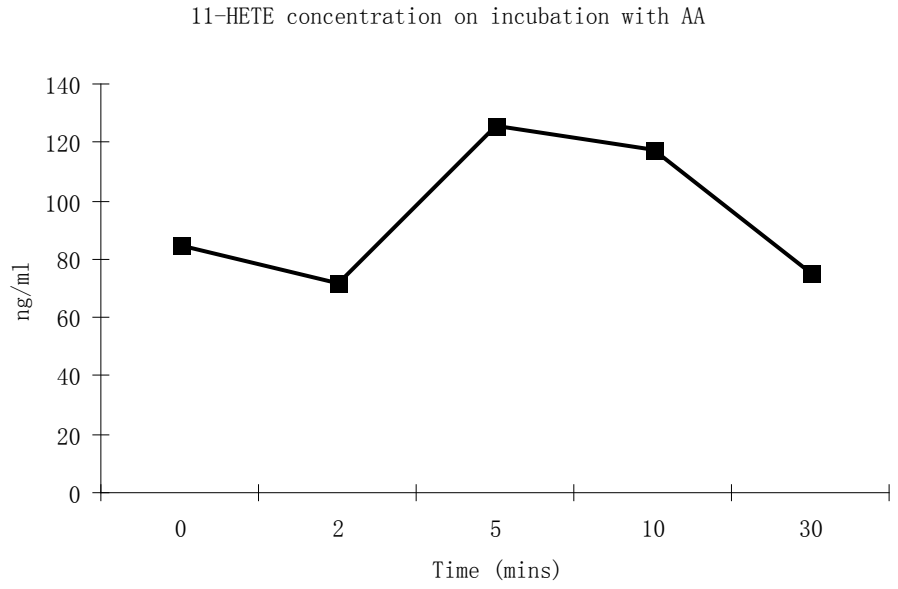


**Figure 4.8 (I)**

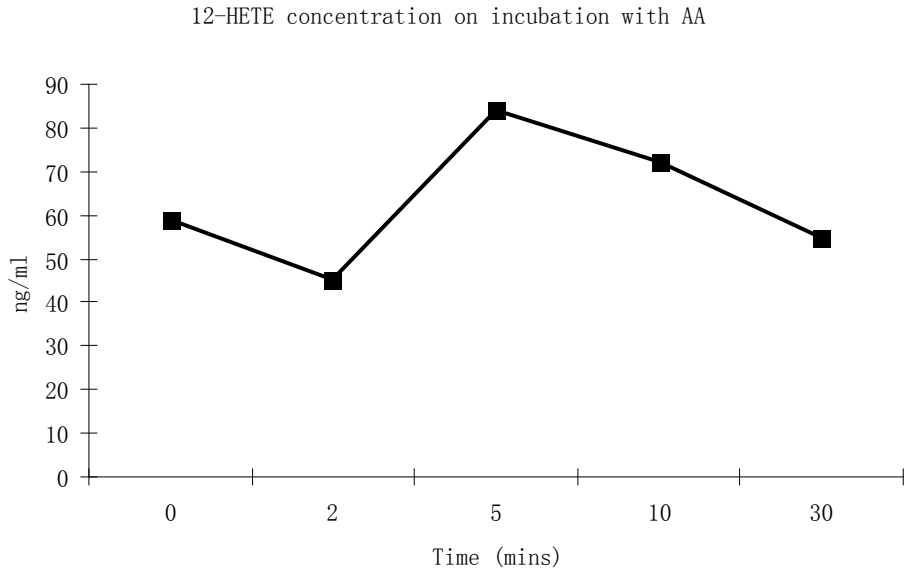


**Figure 4.8 (J)**

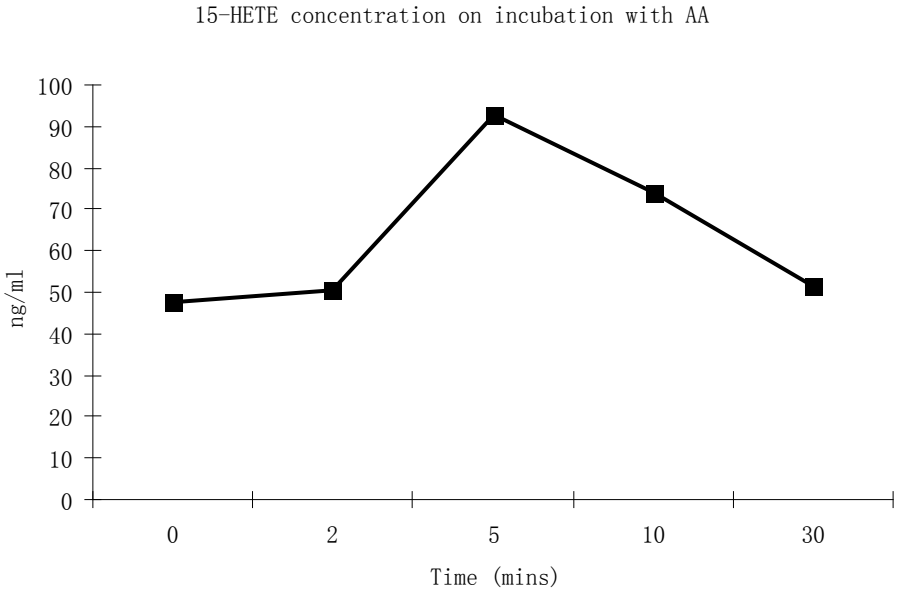


**Figure 4.8 (K)**

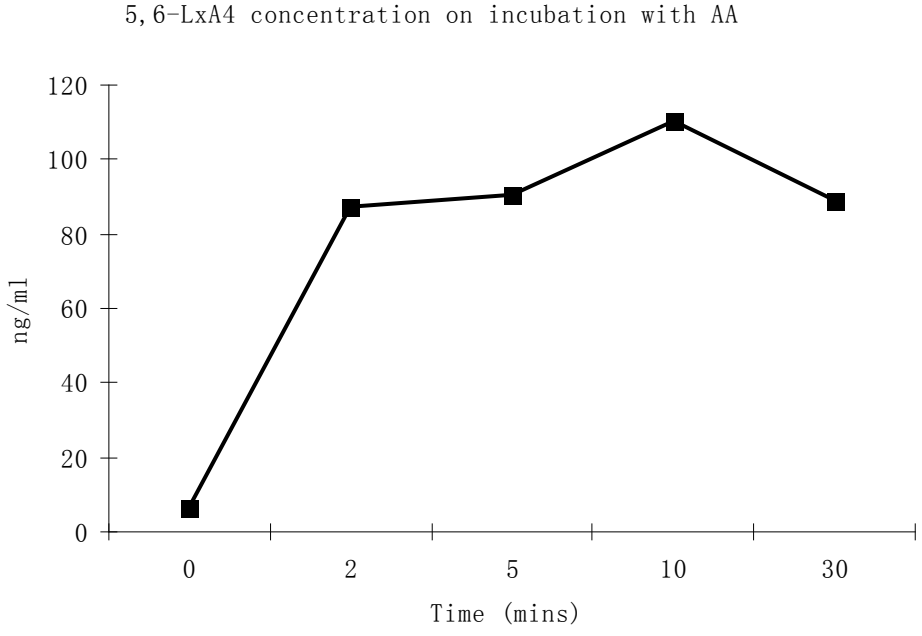


**Figure 4.8 (L)**

**Figure 4.9 (A-A6): Expression of genes involved in eicosanoid production, metabolism and action and in tight junction formation and maintenance.**

HBMECs were incubated in the absence or presence of 0.1 mM AA bound to BSA (1:1 molar ratio) for up to 30 minutes. Total RNA was isolated and mRNA expression of 32 genes determined as described in Materials and Methods. Data represents the mean ± SE of three experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

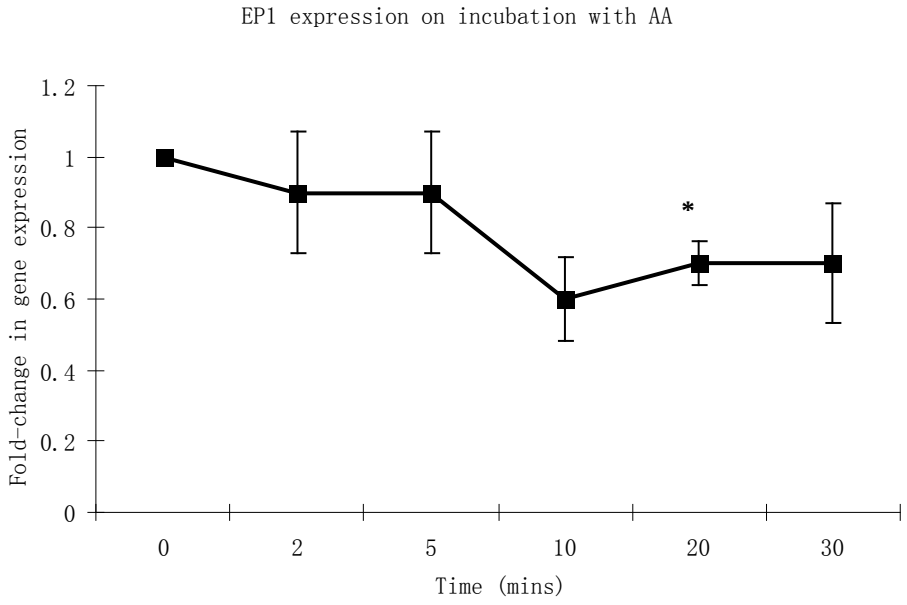


**Figure 4.9 (A)**

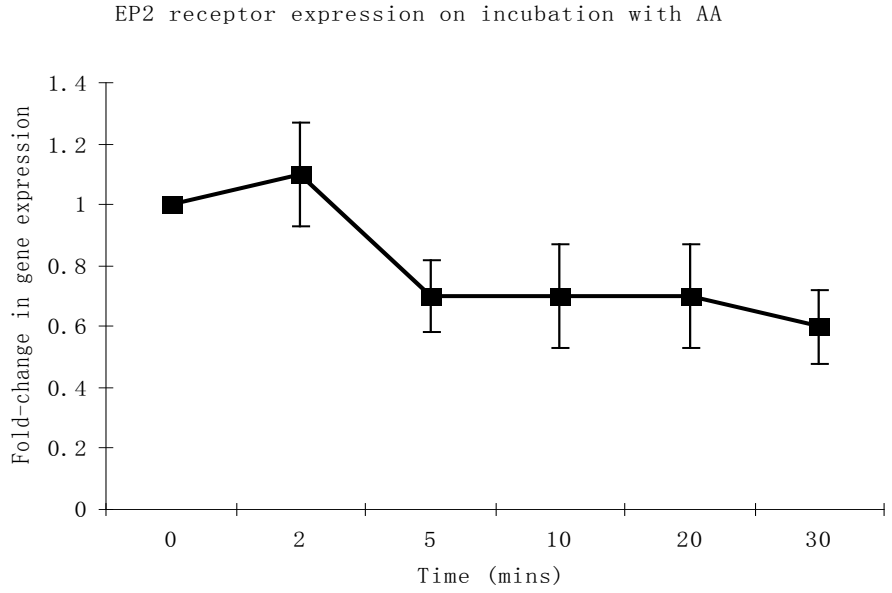


**Figure 4.9 (B)**

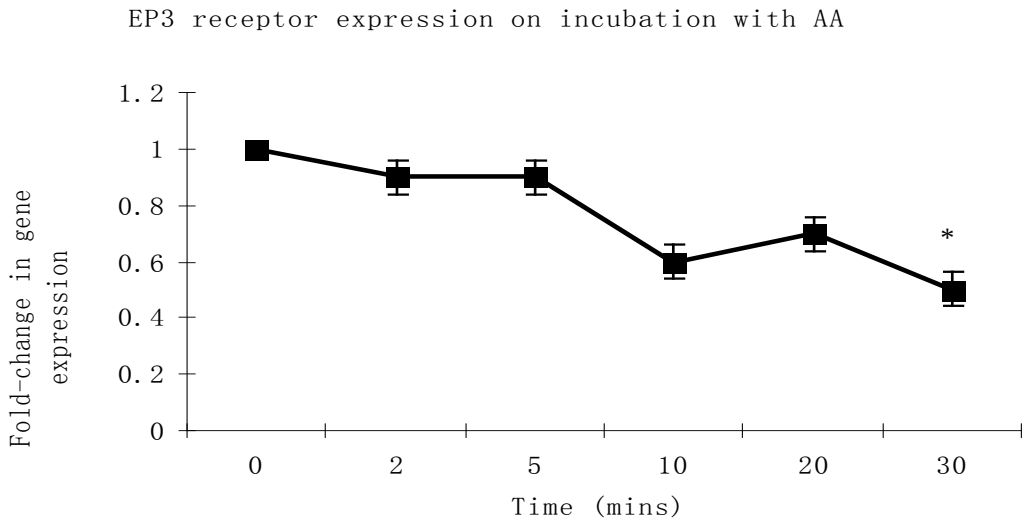


**Figure 4.9 (C)**

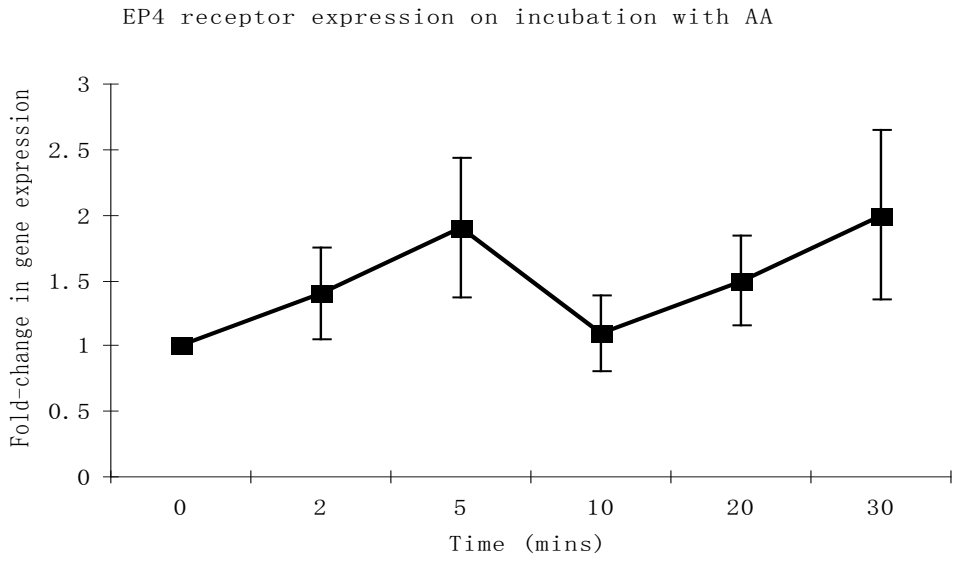


**Figure 4.9 (D)**

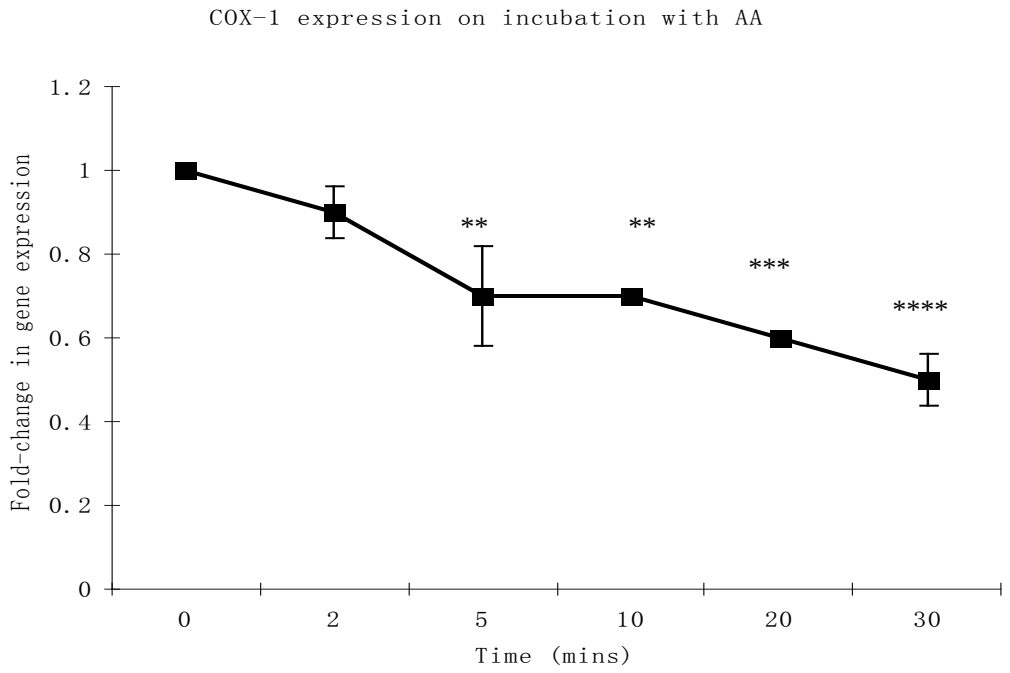


**Figure 4.9 (E)**

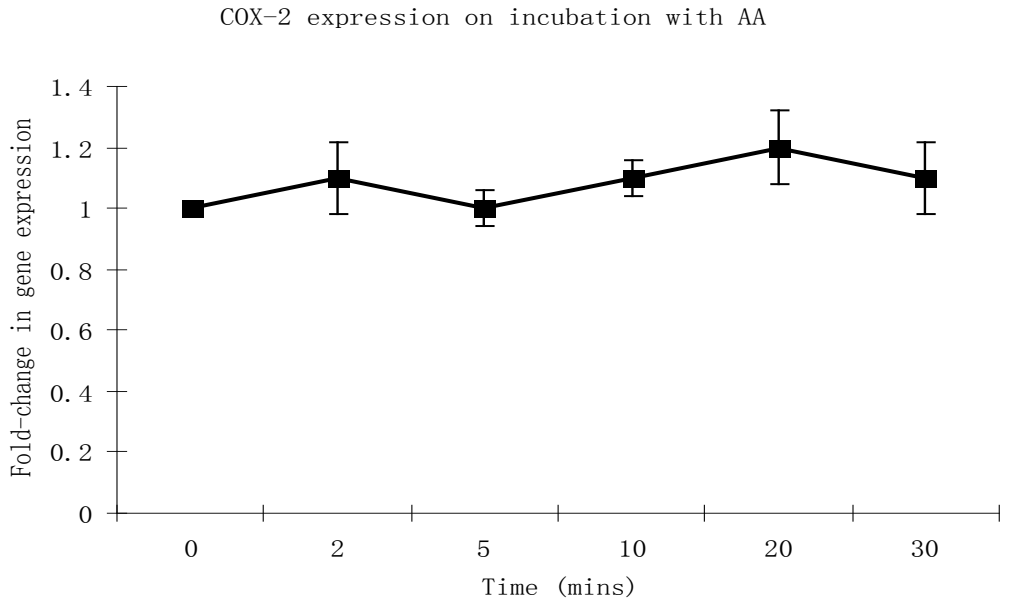


**Figure 4.9 (F)**

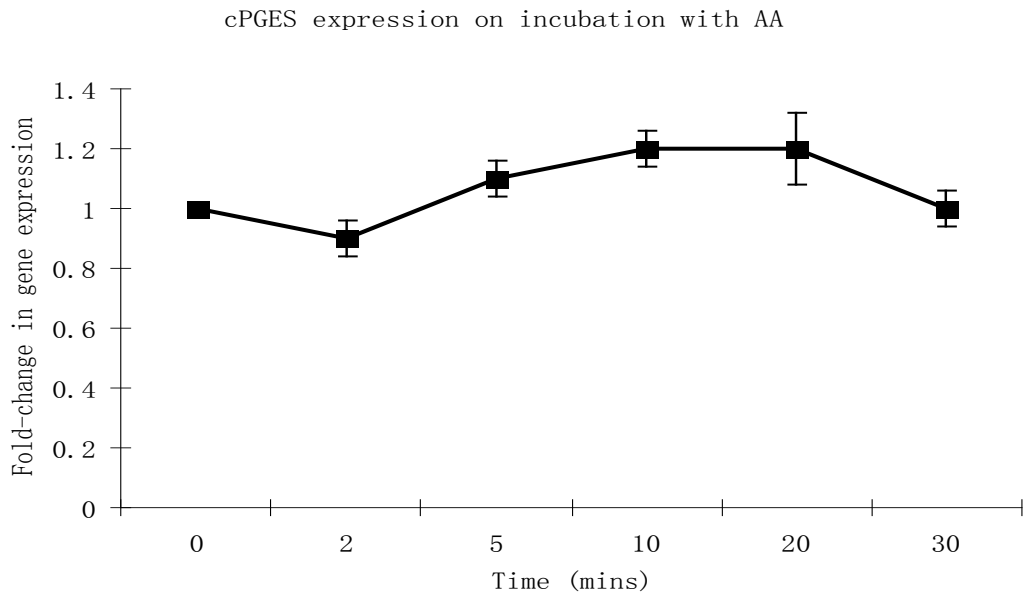


**Figure 4.9 (G)**

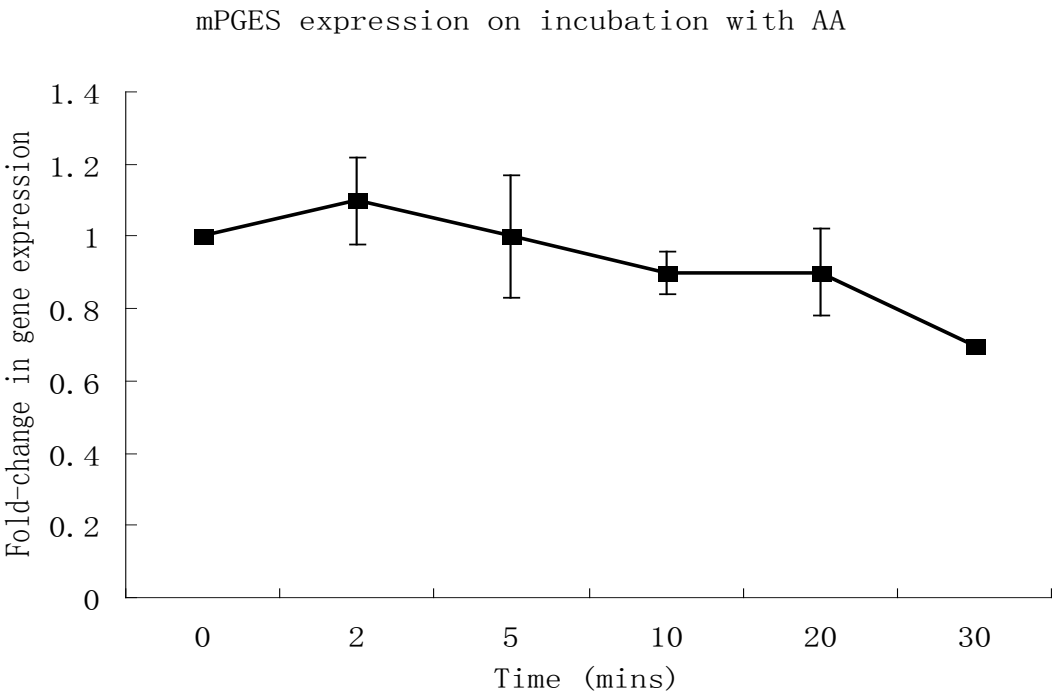


**Figure 4.9 (H)**

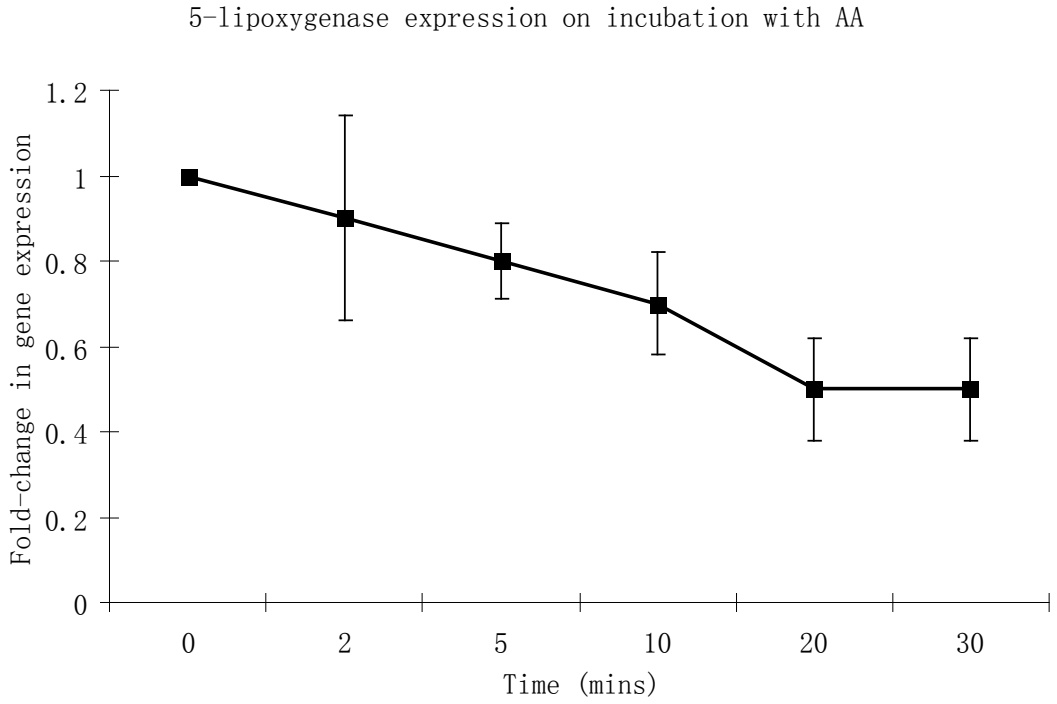


**Figure 4.9 (I)**

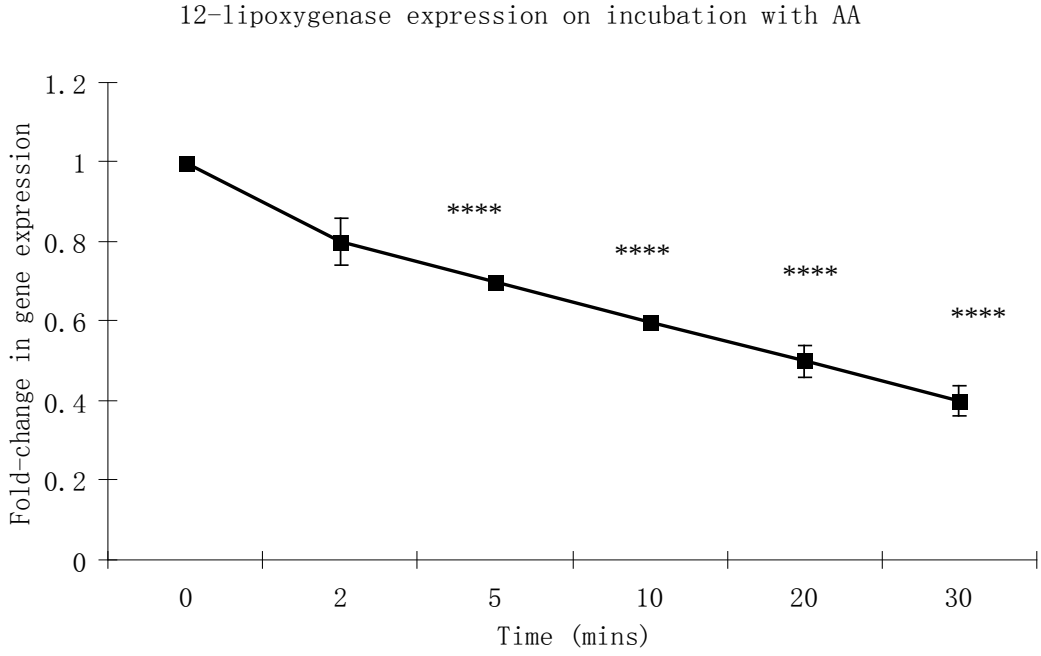


**Figure 4.9 (J)**

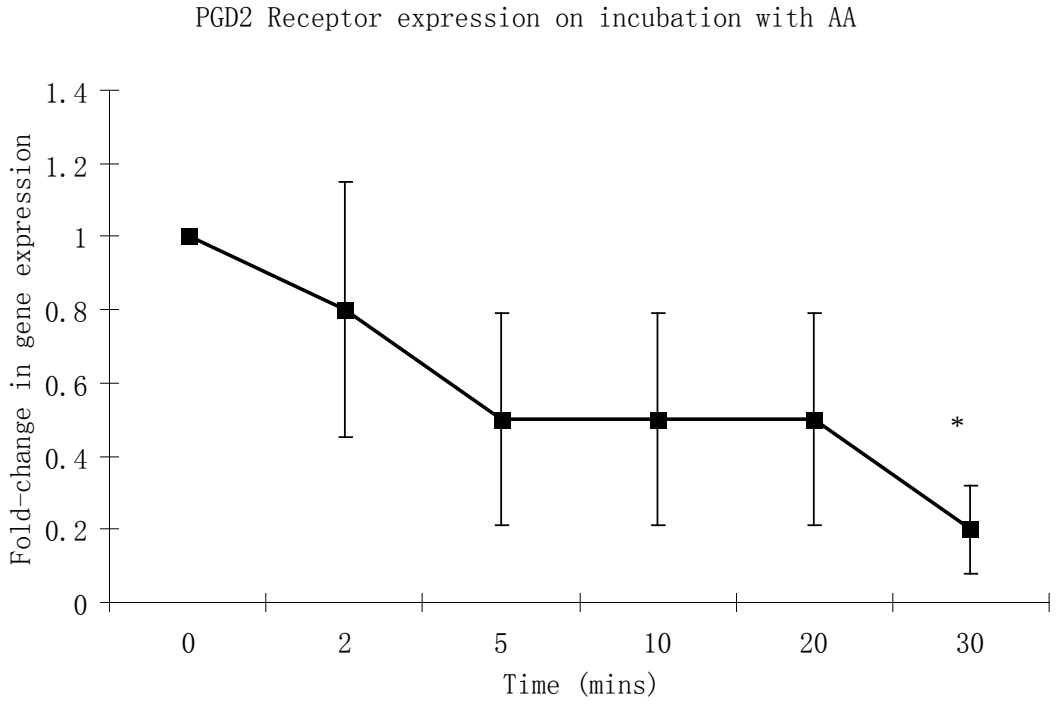


**Figure 4.9 (K)**

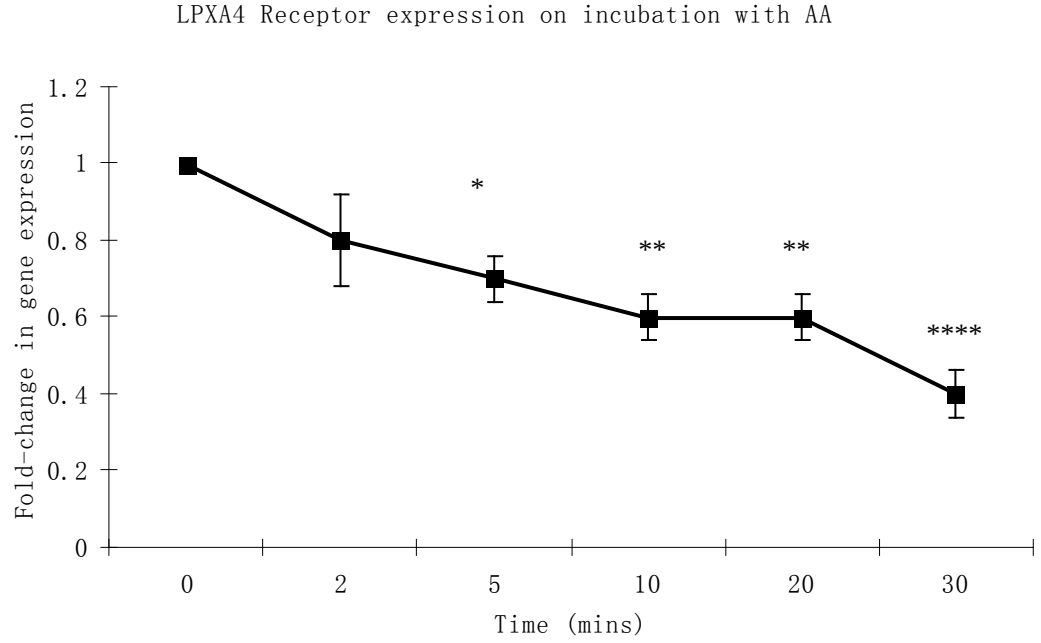


**Figure 4.9 (L)**

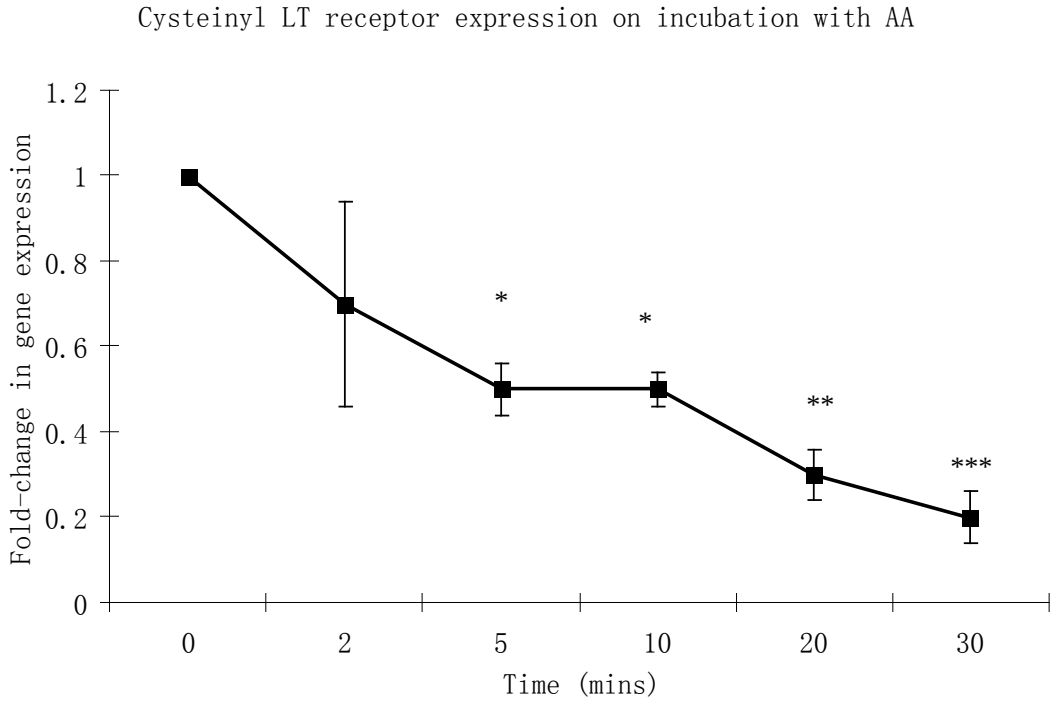


**Figure 4.9 (M)**

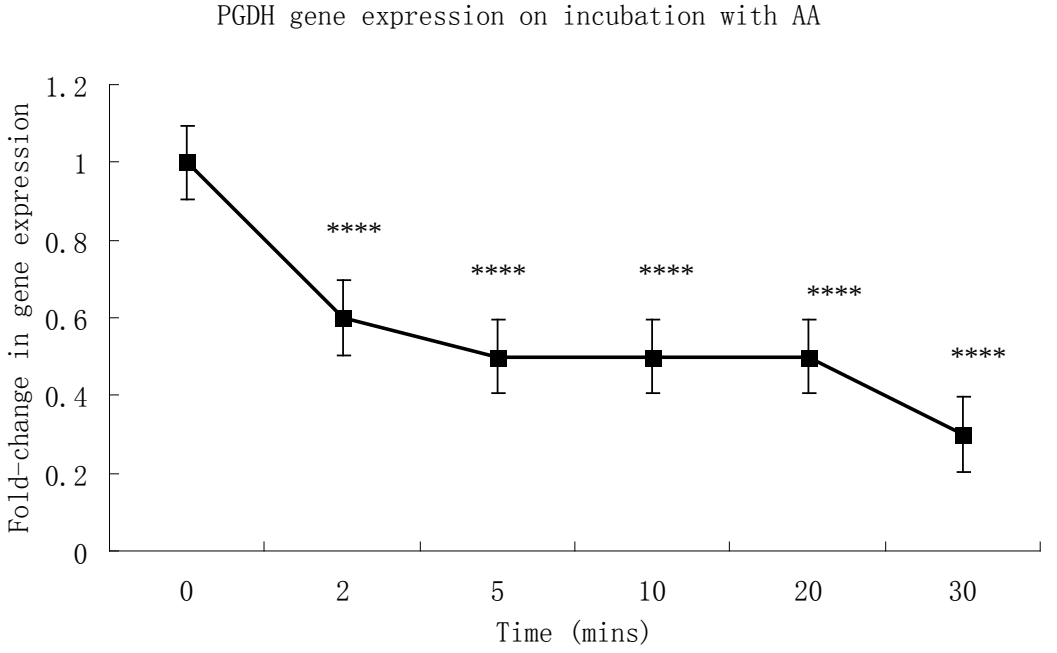


**Figure 4.9 (N)**

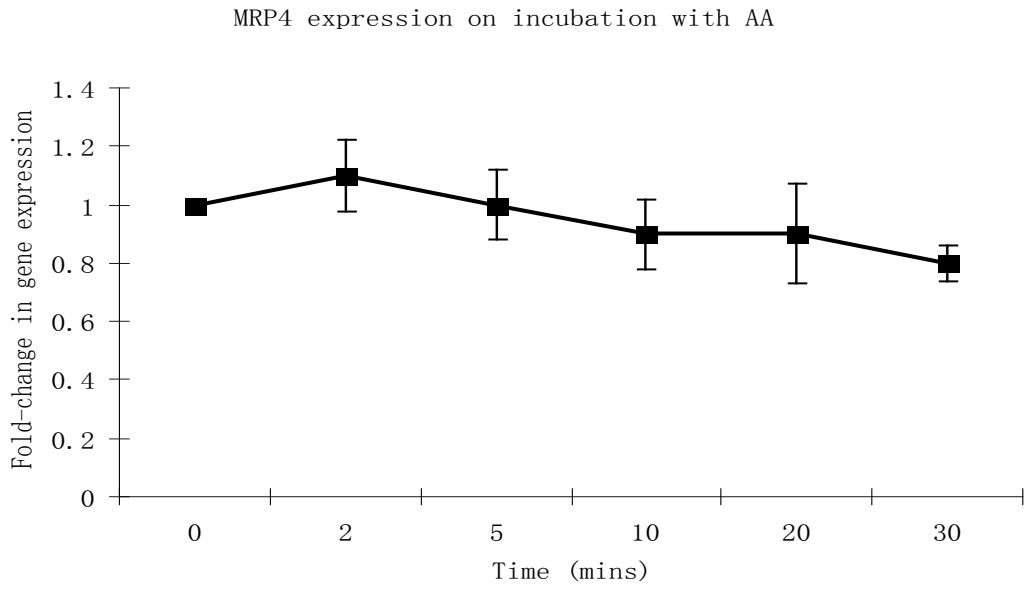


**Figure 4.9 (O)**

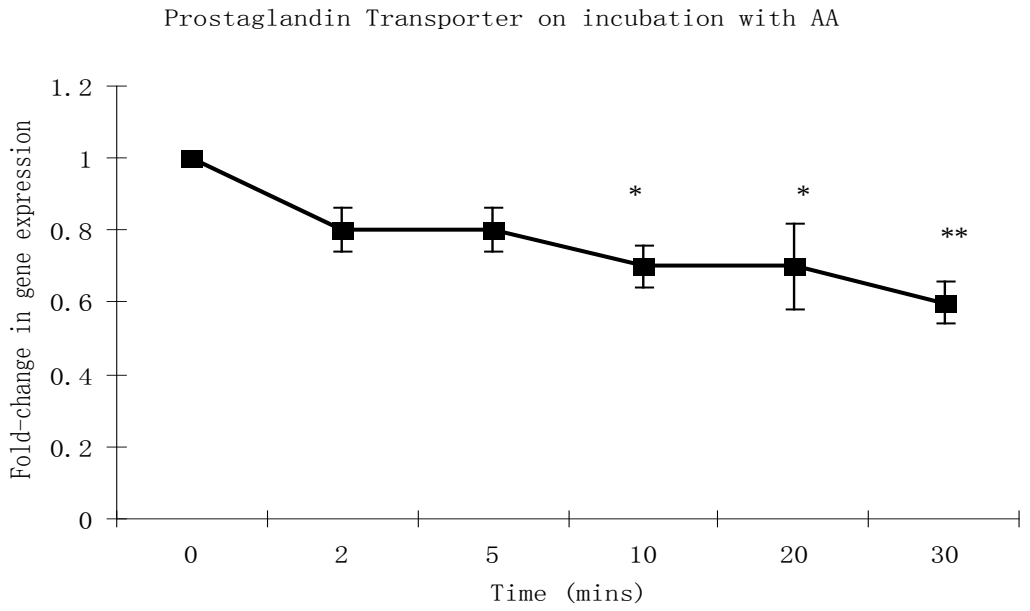


**Figure 4.9 (P)**

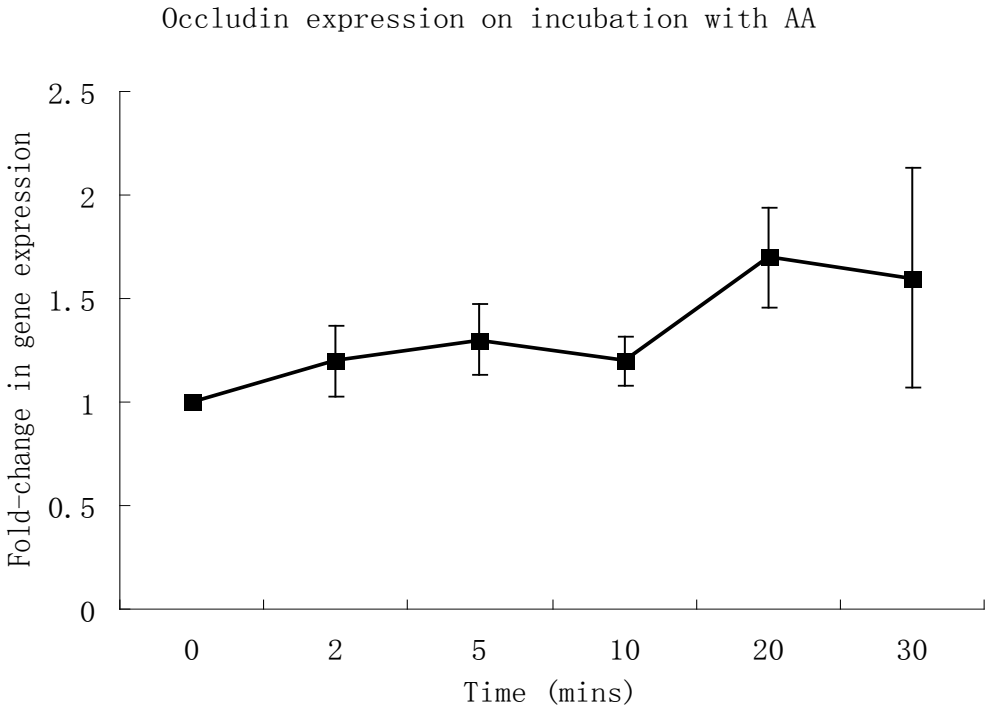


**Figure 4.9 (Q)**

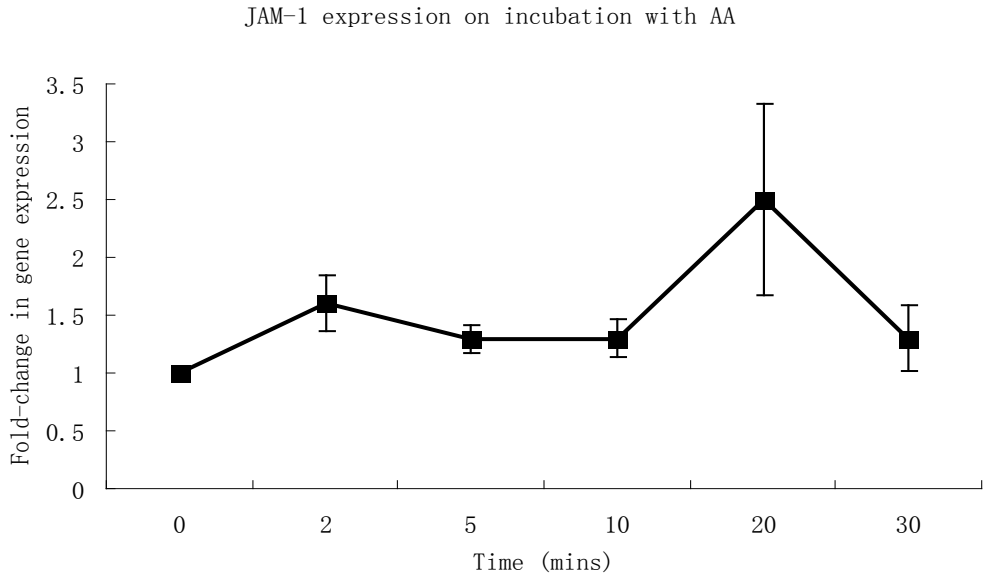


**Figure 4.9 (R)**

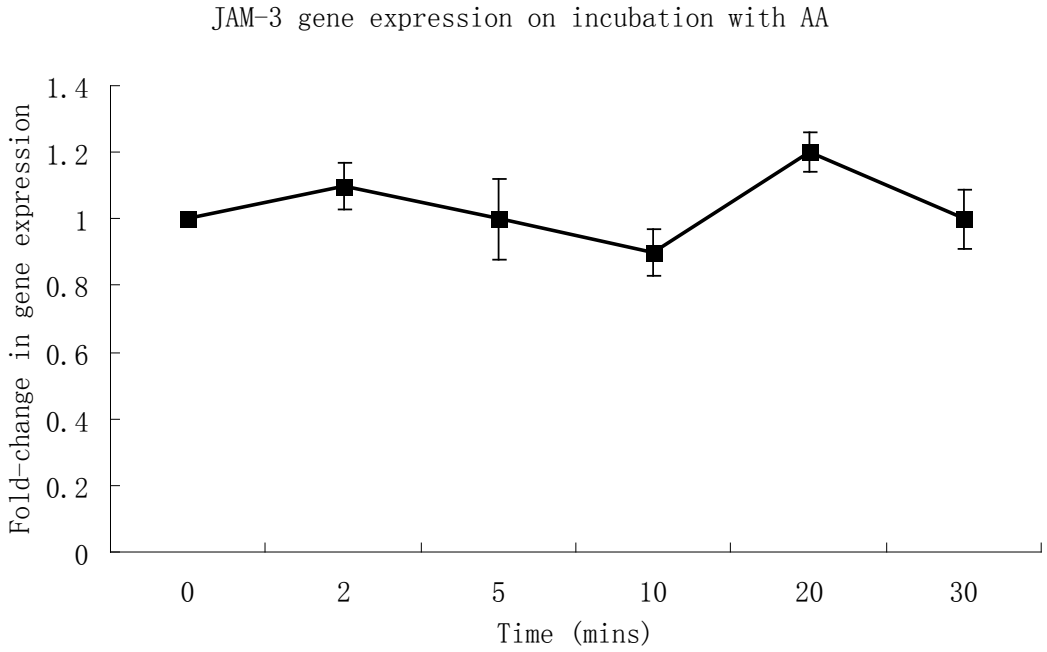


**Figure 4.9 (S)**

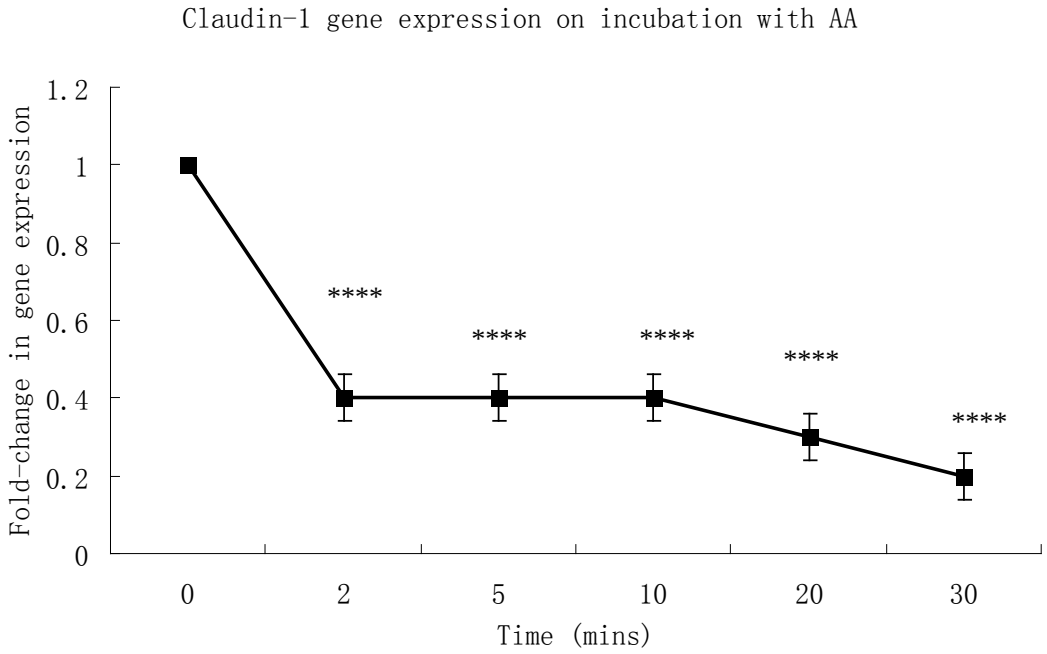


**Figure 4.9 (T)**

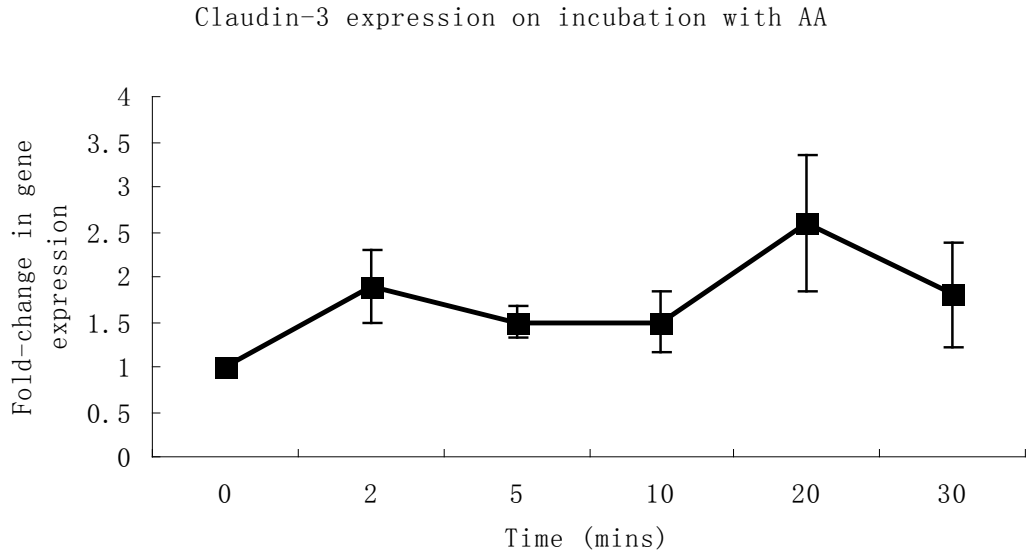


**Figure 4.9 (U)**

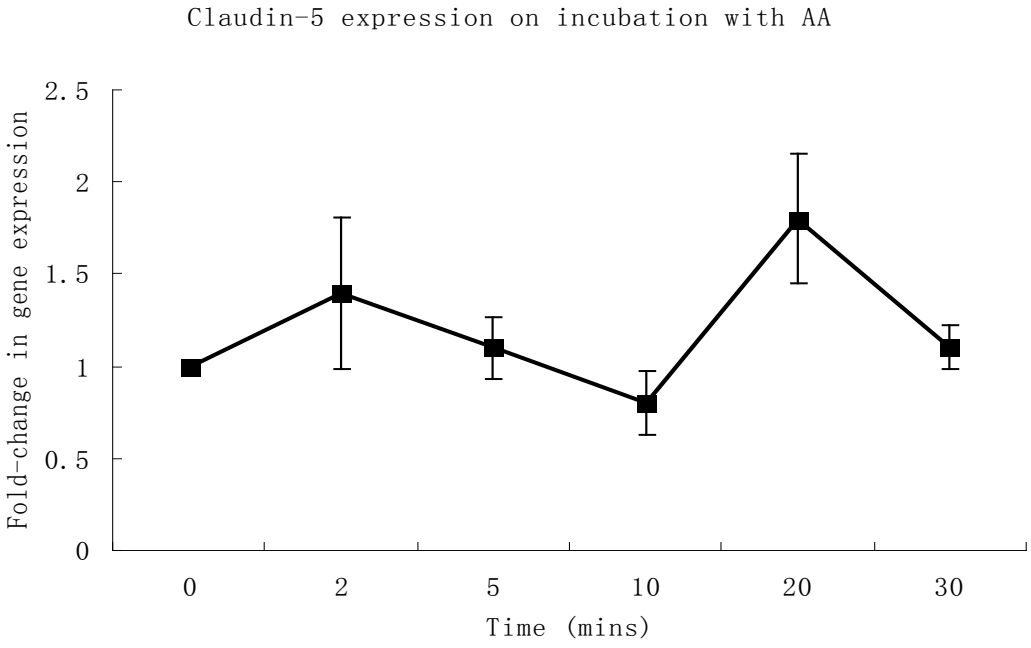


**Figure 4.9 (V)**

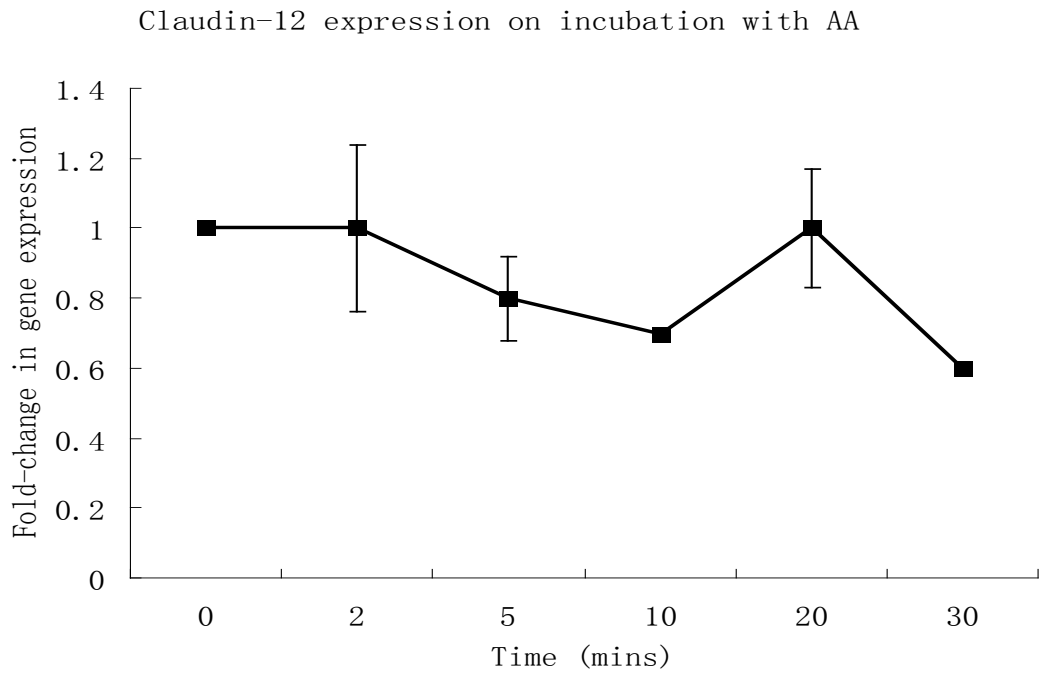


**Figure 4.9 (W)**

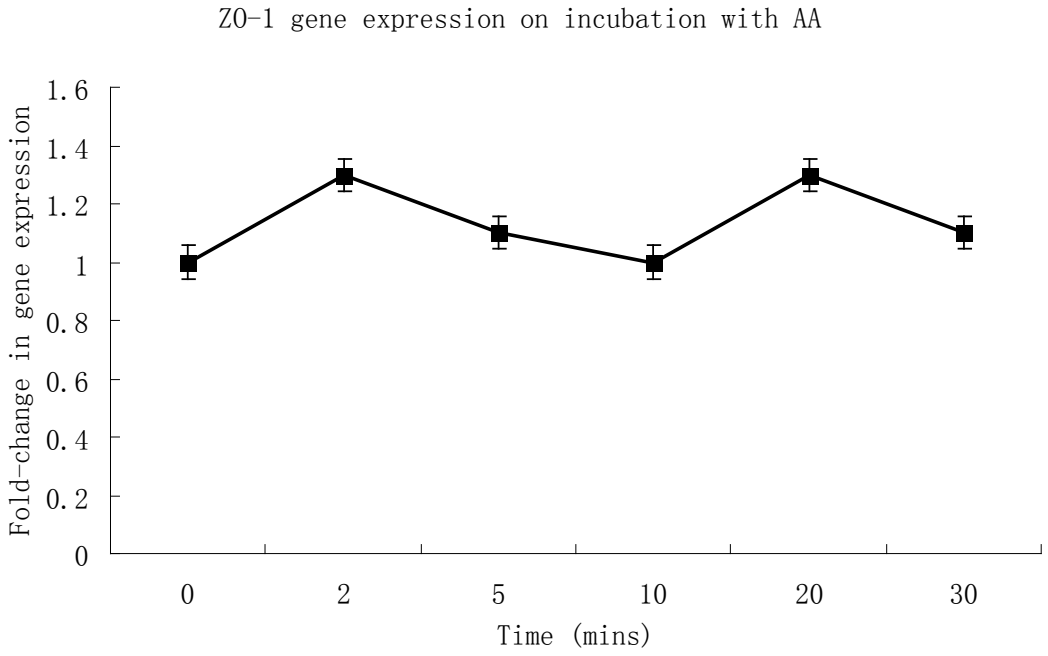


**Figure 4.9 (X)**

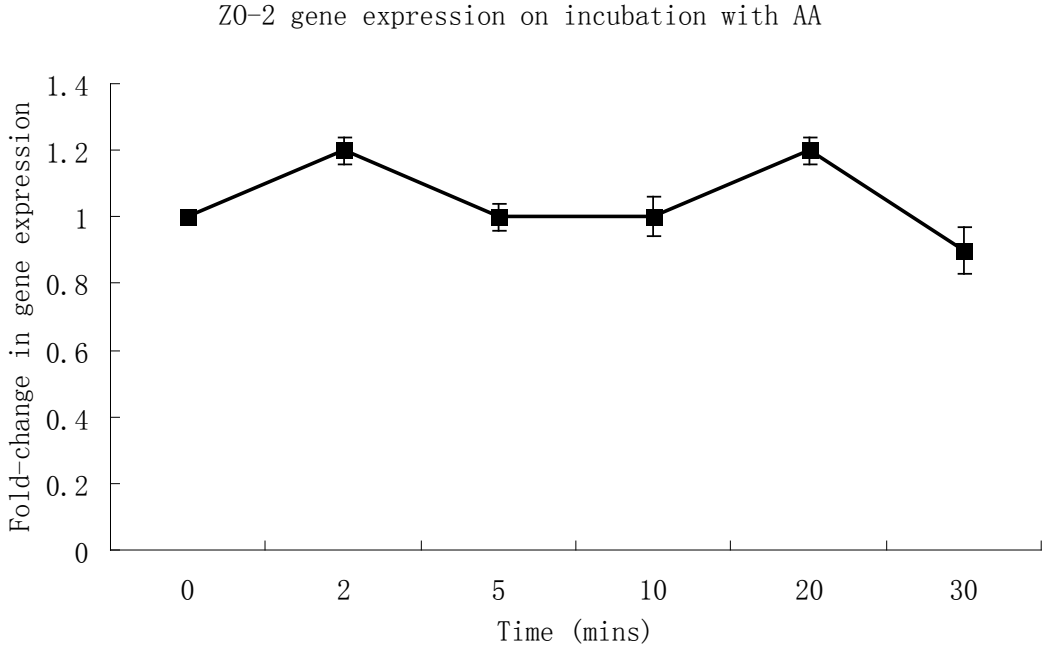


**Figure 4.9 (Y)**

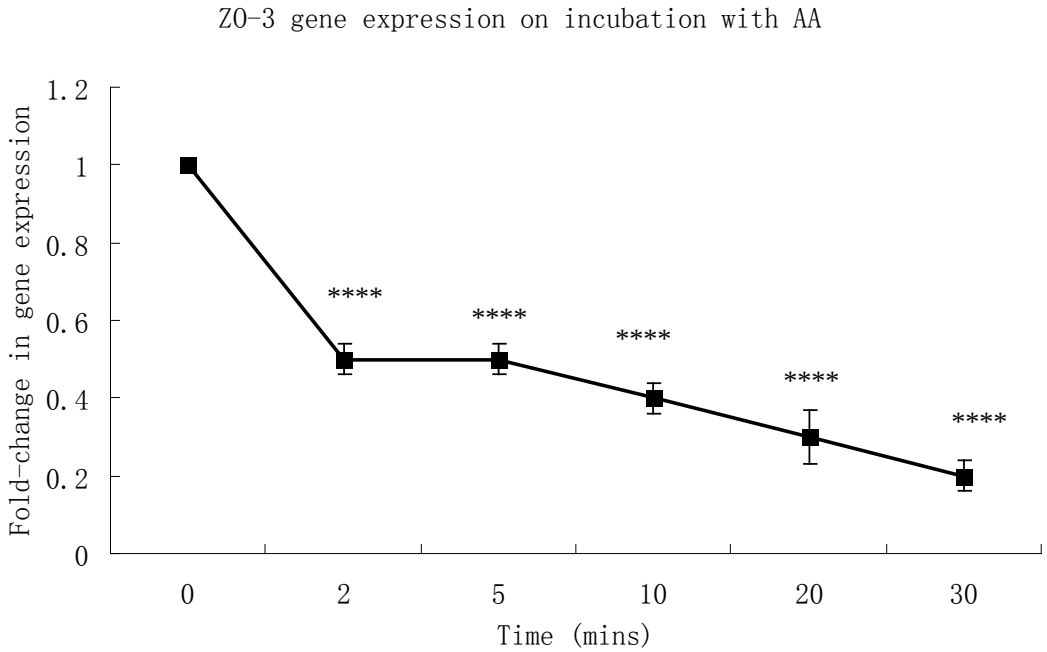


**Figure 4.9 (Z)**

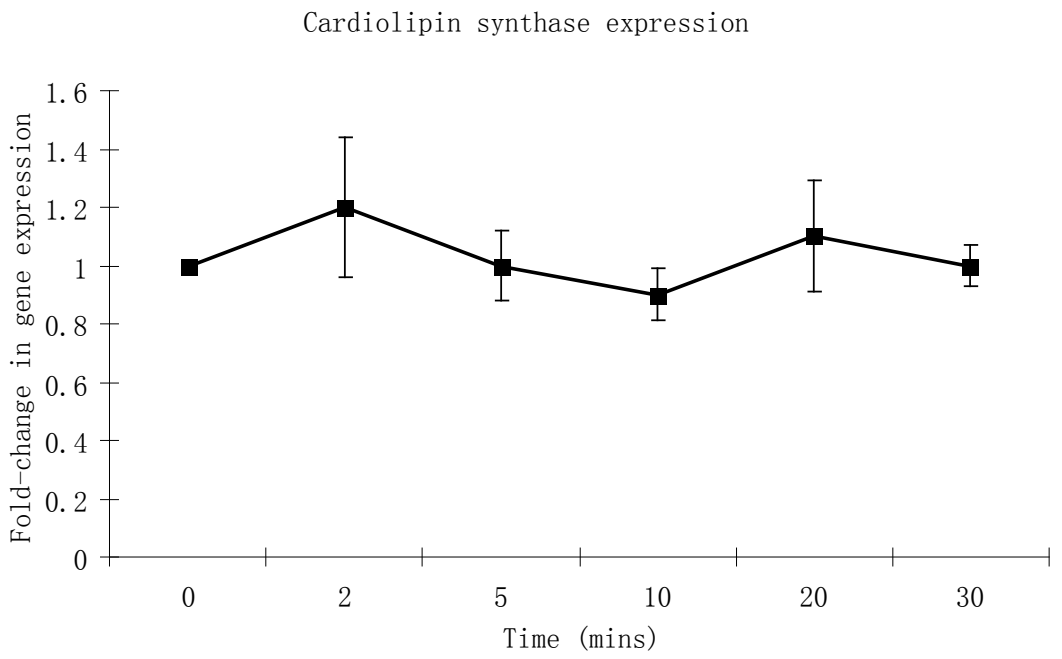


**Figure 4.9 (A1)**

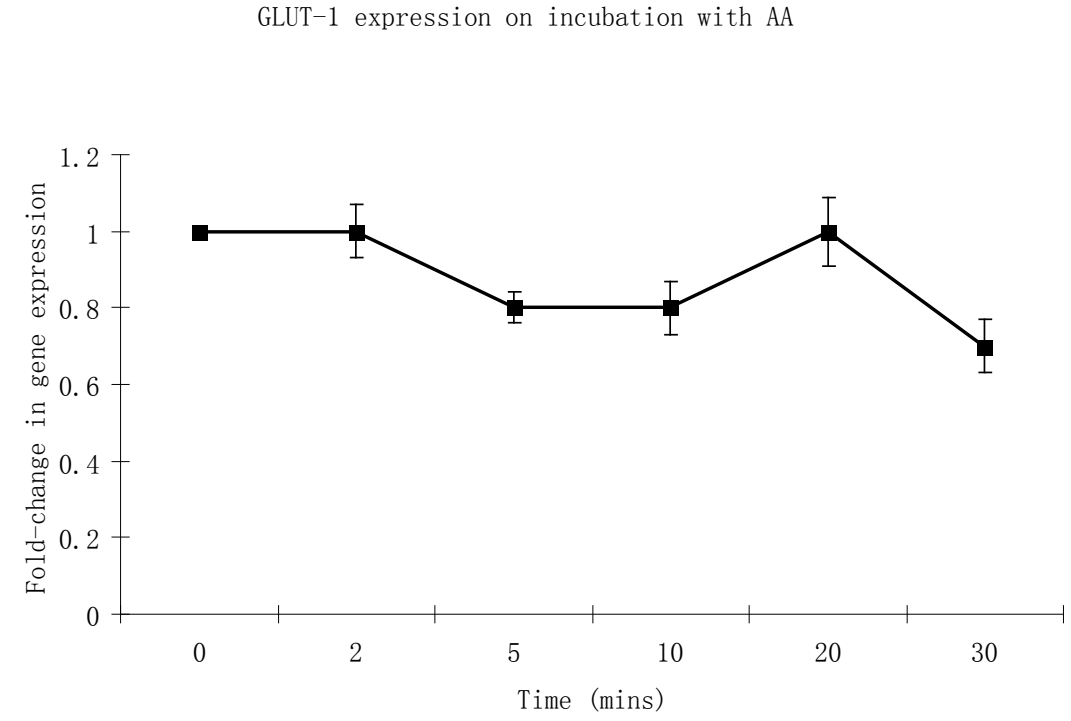


**Figure 4.9 (A2)**

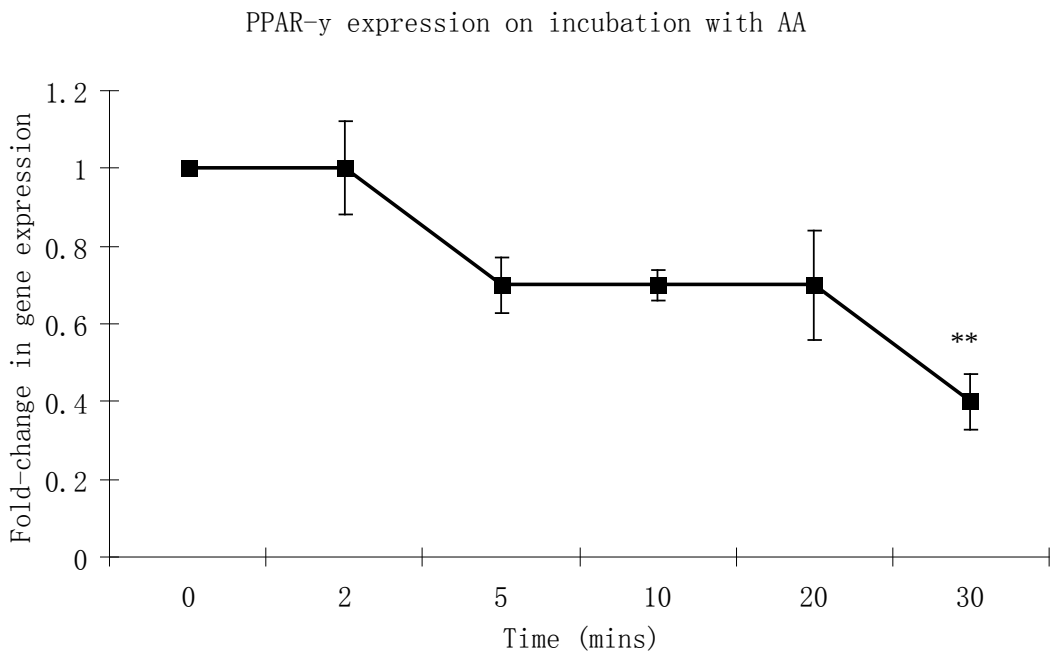


**Figure 4.9 (A3)**

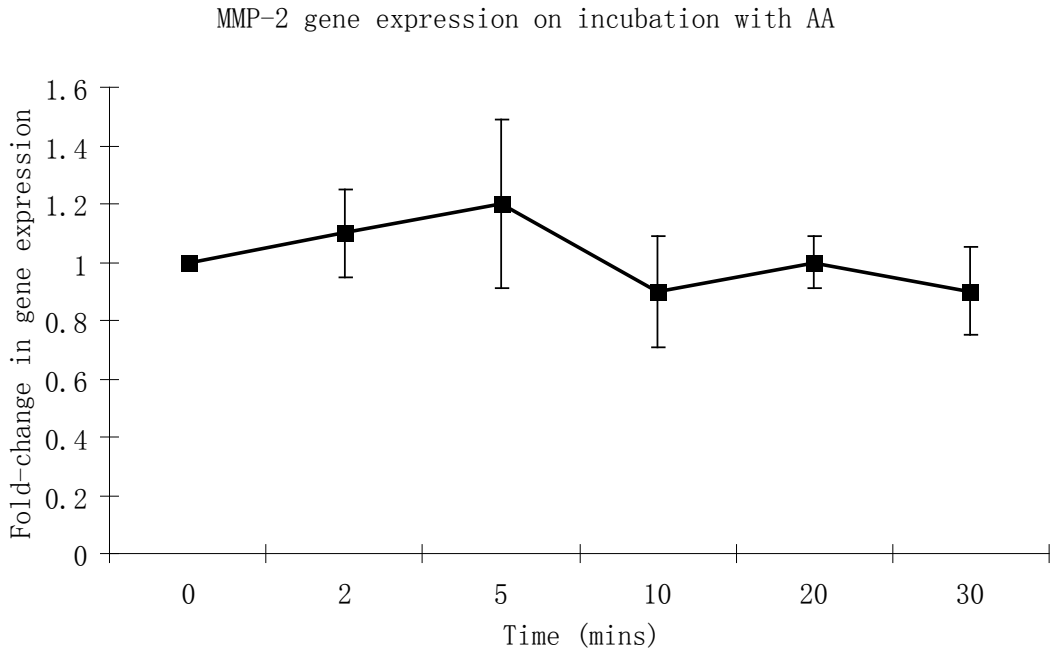


**Figure 4.9 (A4)**

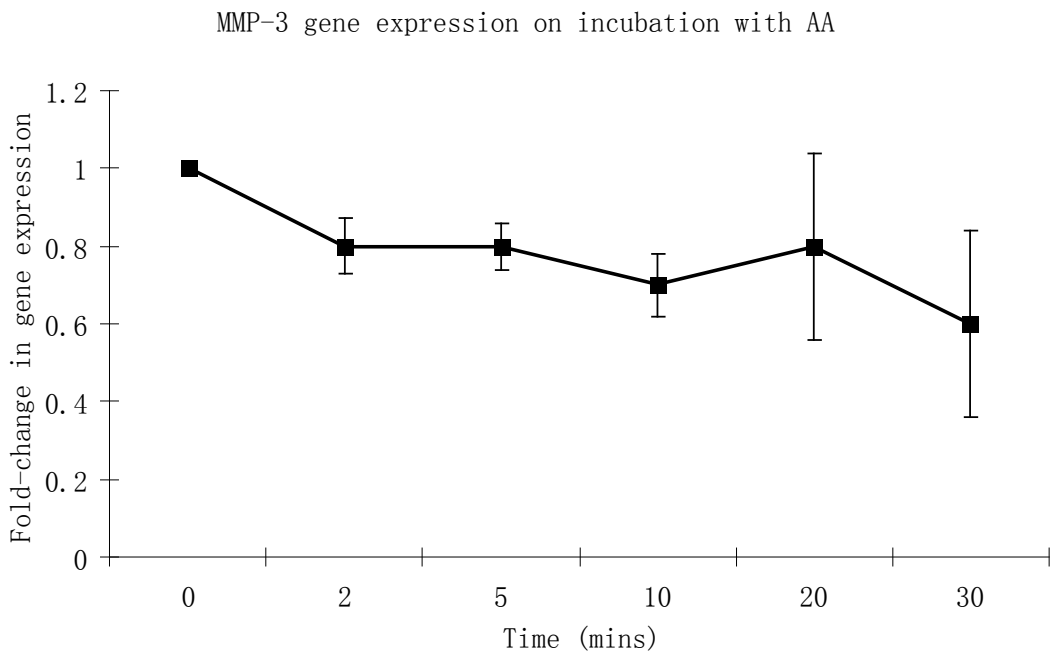


**Figure 4.9 (A5)**

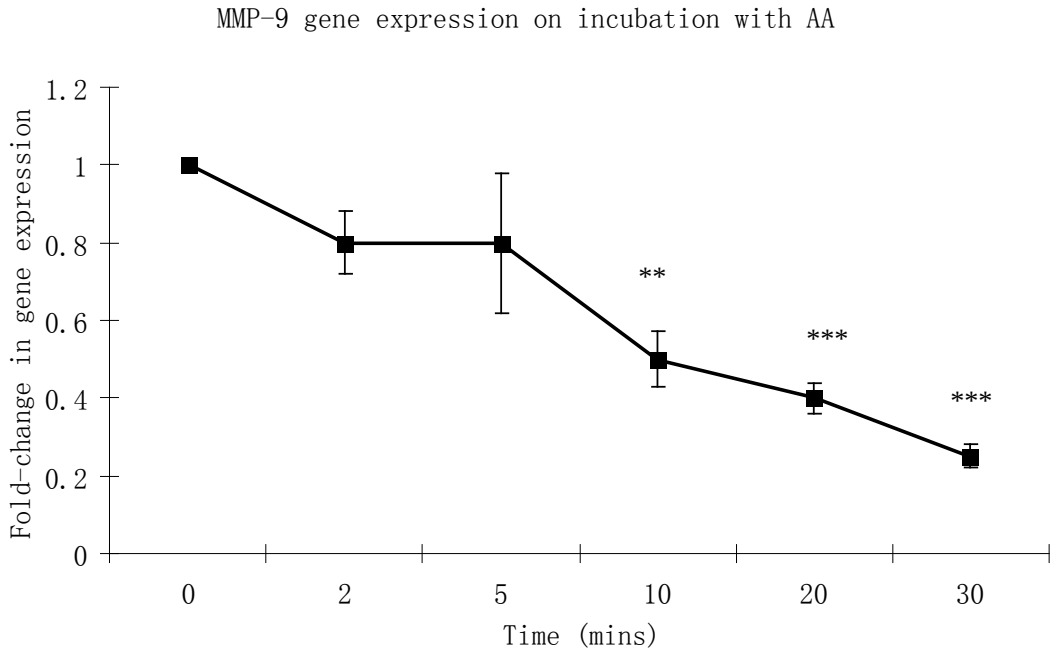


**Figure 4.9 (A6)**

**Figure 4.10: Incubation of HBMEC with EPA or DHA does not alter FDX flux across HBMEC monolayers.**

HBMECs were incubated with 0.1 mM EPA (Fig. 4.10 A) or DHA (Fig. 4.10 B) bound to BSA (1:1 molar ratio) and 1 μM FDX. FDX flux into the basolateral medium determined as described in Materials and Methods. Data represents the mean of three experiments.

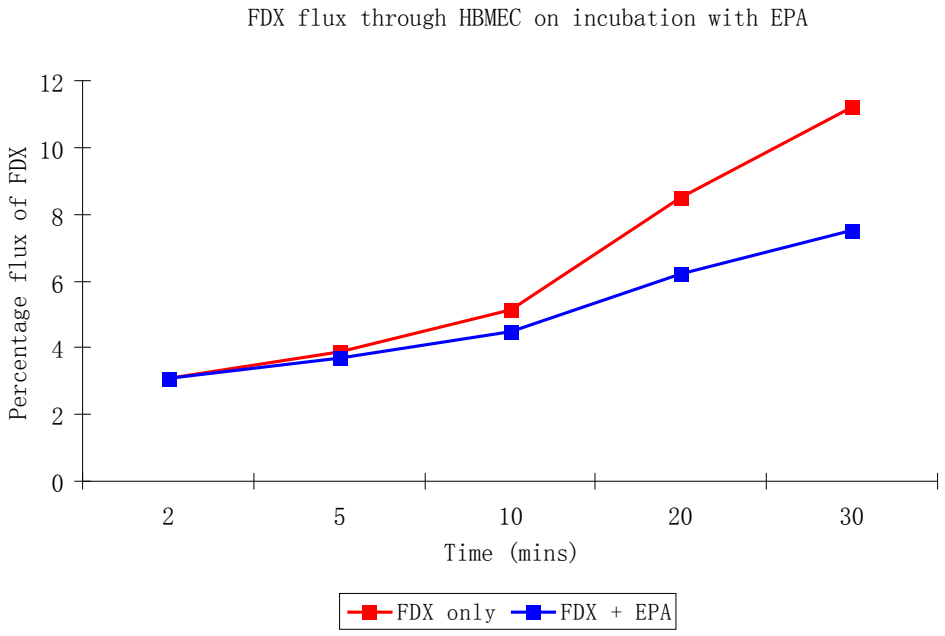


**Figure 4.10 (A)**

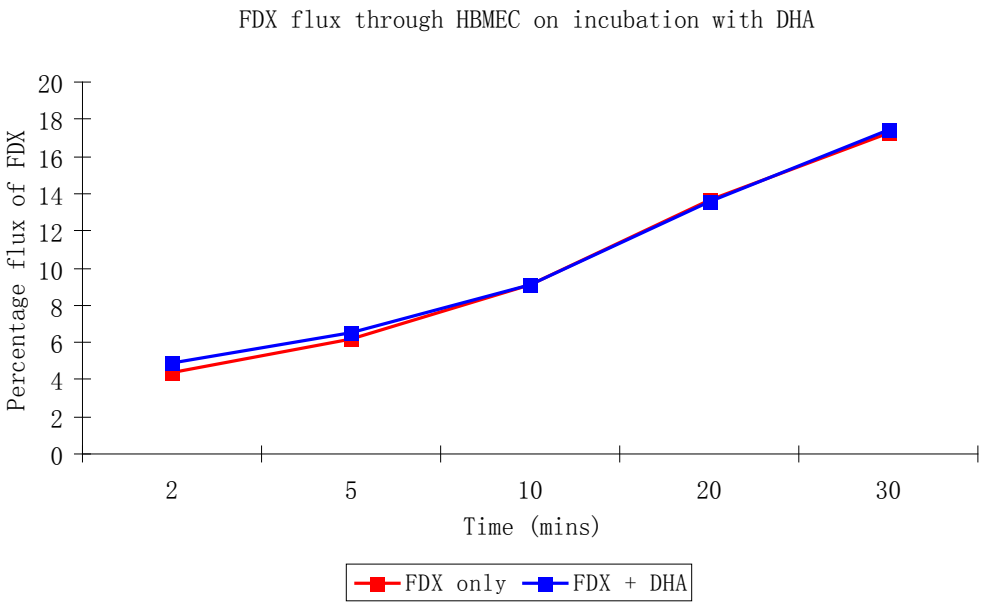
FDX flux through HBMEC on incubation with DHA
Percentage flux of FDX
20
18
16
14
12
10
8
6
4
2
0
2
5
10
20
30
Time (mins)
FDX only
FDX + DHA

**Figure 4.10 (B)**

**Figure 4.11: EPA metabolites generated by HBMEC.**

HBMECs were incubated in the absence or presence of 0.1 mM EPA in complete cell culture medium for up to 30 minutes and EPA metabolites determined by mass spectrometry analysis.

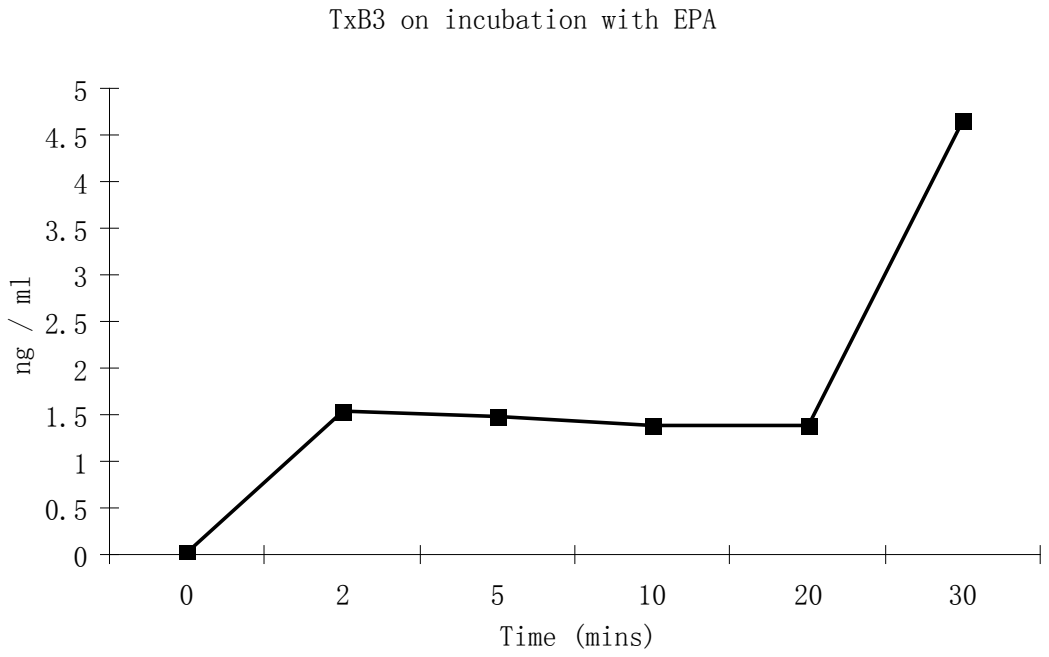


**Figure 4.11 (A)**

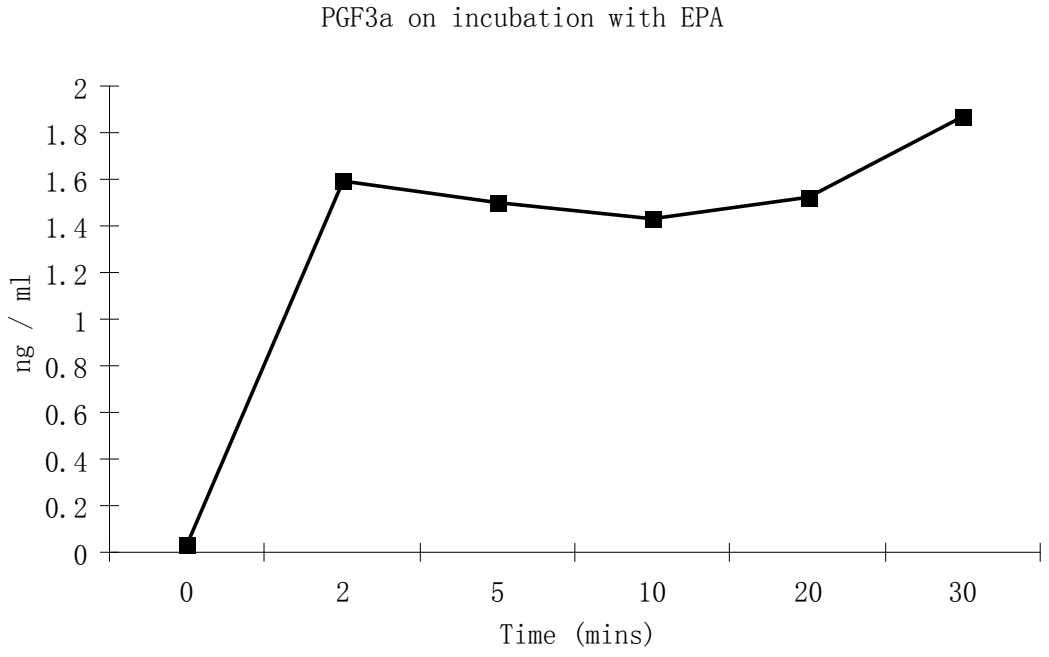


**Figure 4.11 (B)**

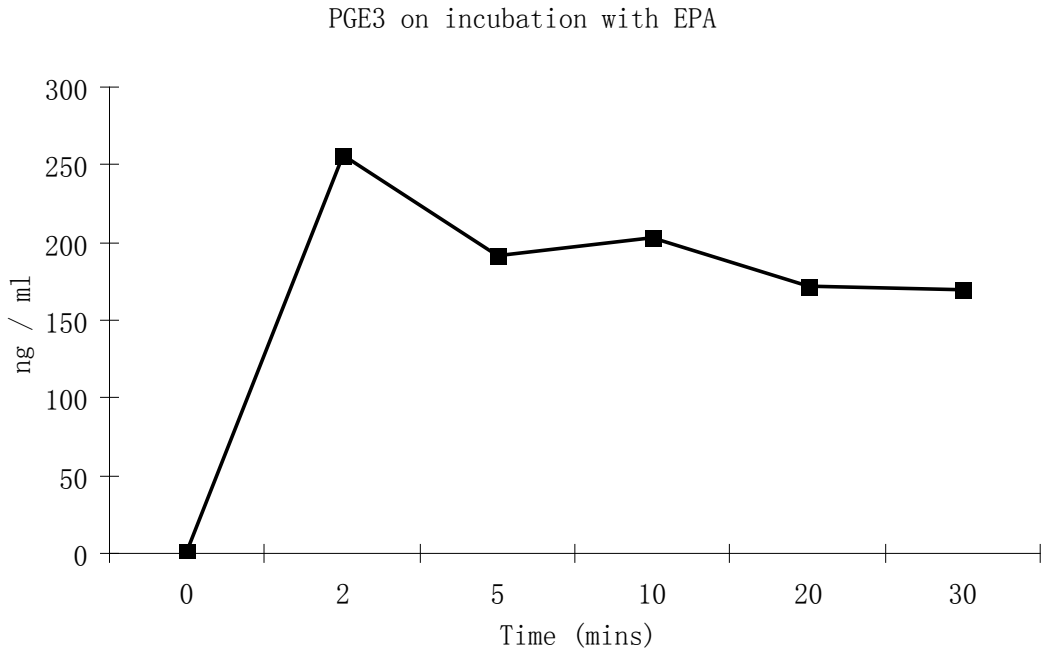


**Figure 4.11 (C)**

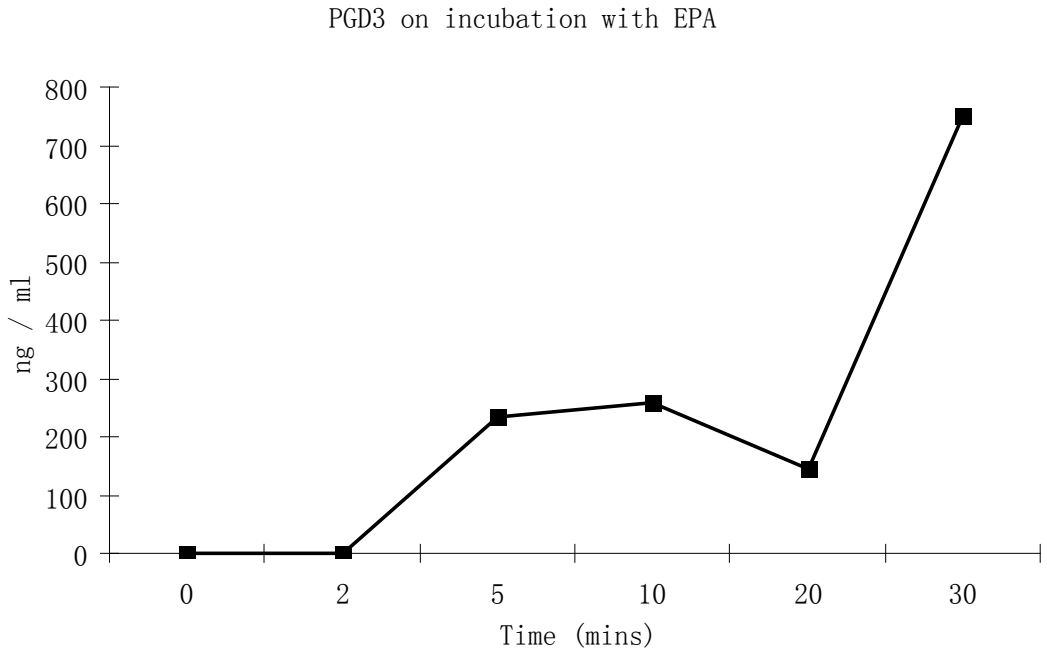


**Figure 4.11 (D)**

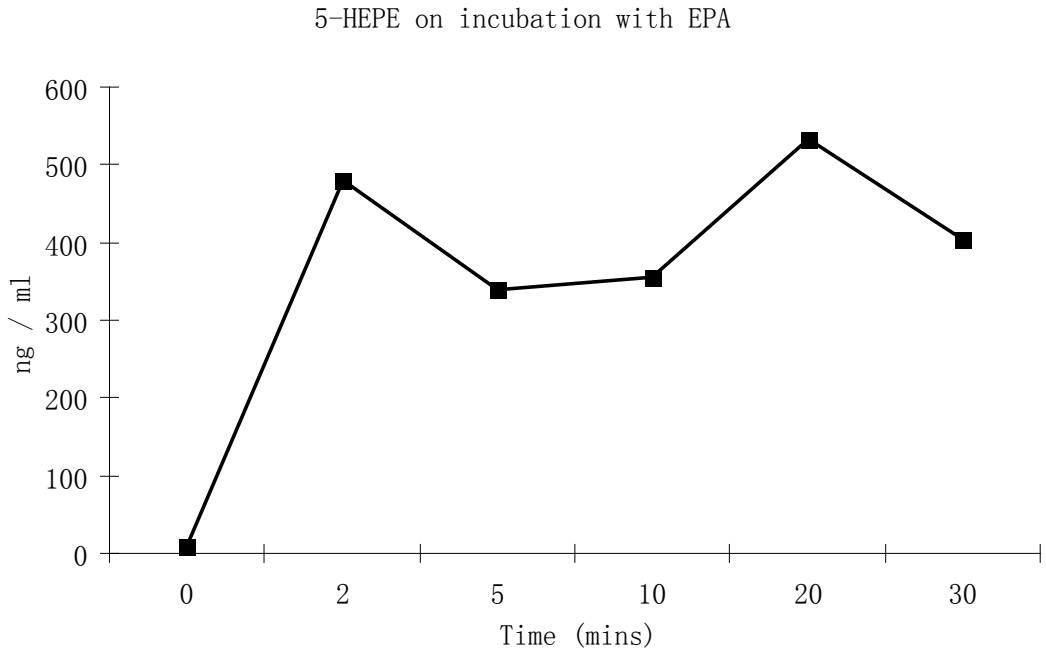


**Figure 4.11 (E)**

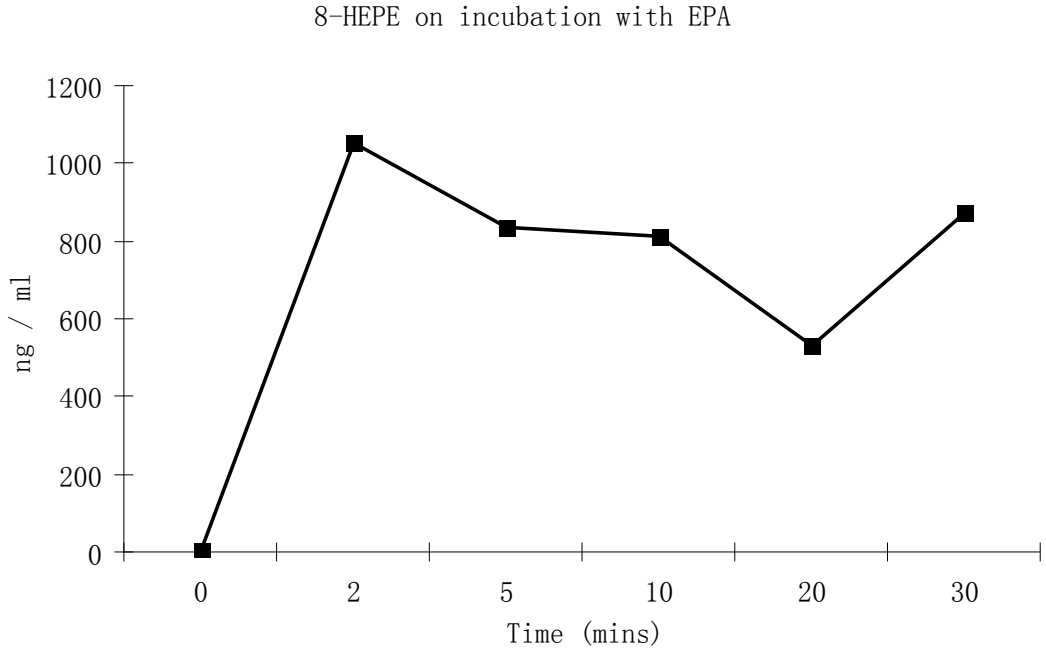


**Figure 4.11 (F)**

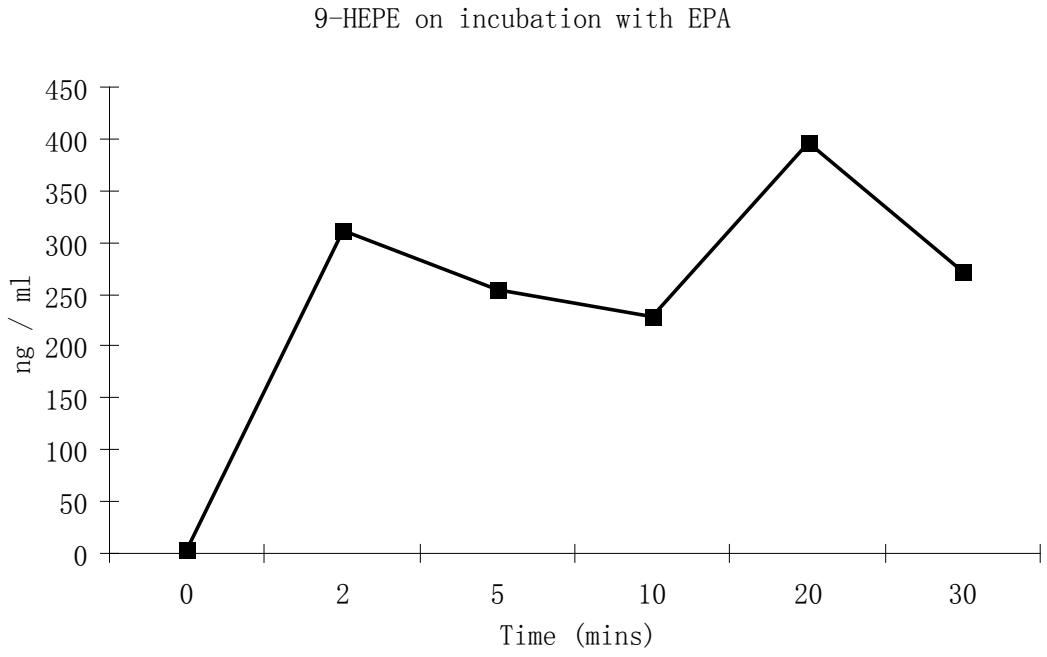


**Figure 4.11 (G)**

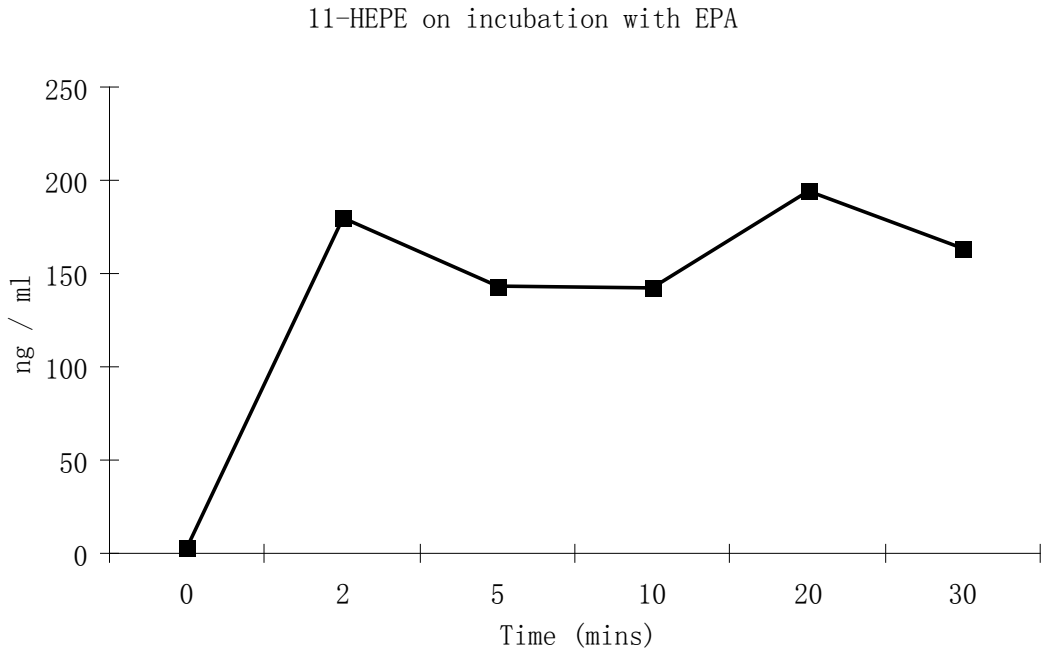


**Figure 4.11 (H)**

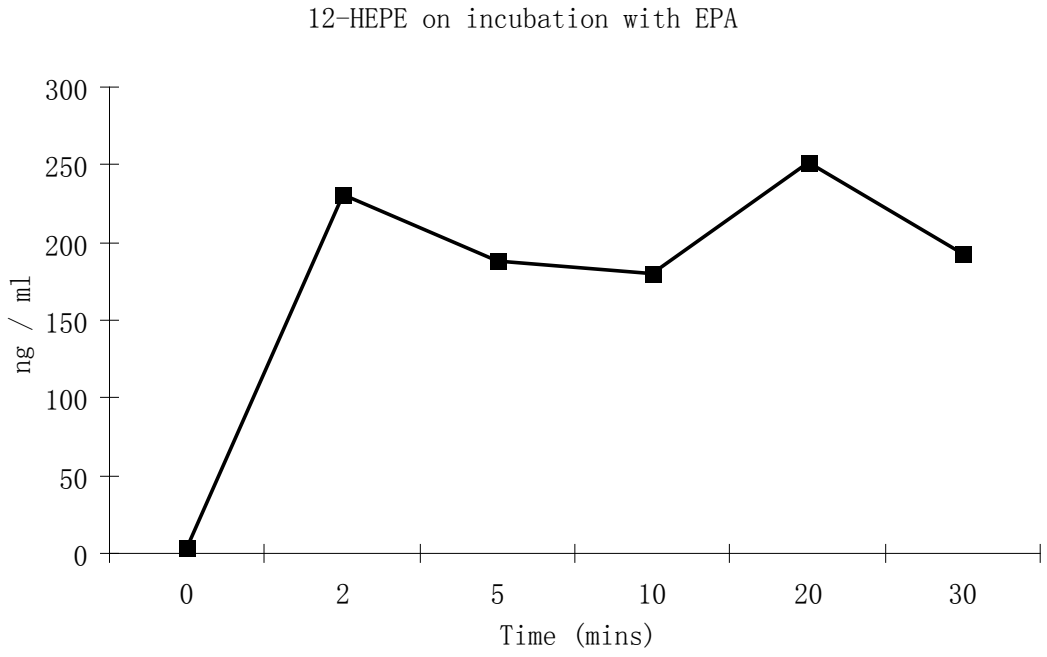


**Figure 4.11 (I)**

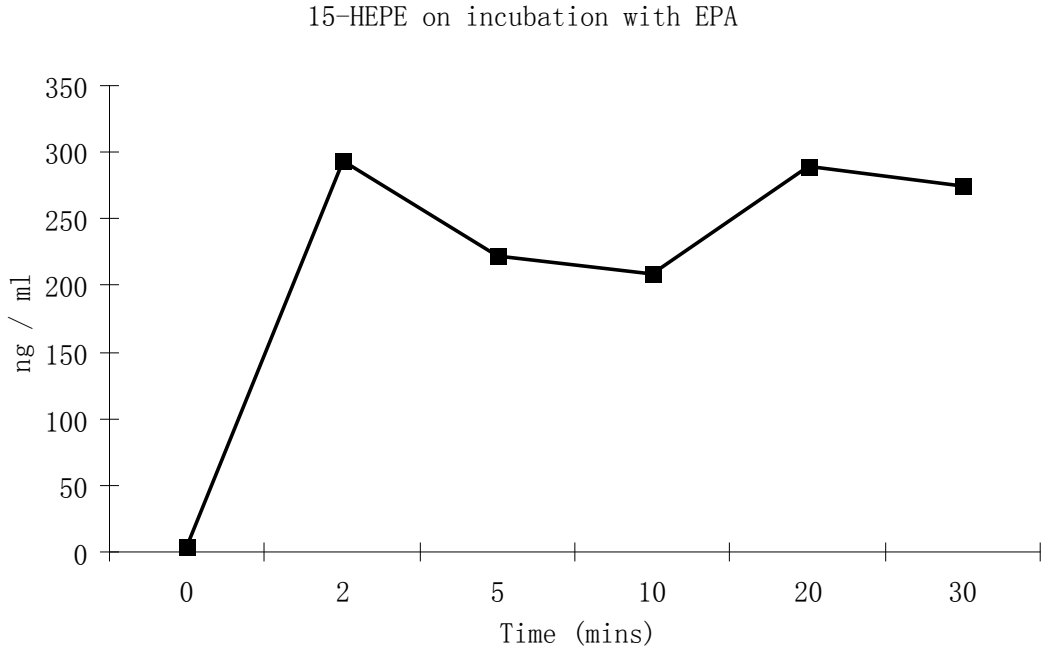


**Figure 4.11 (J)**

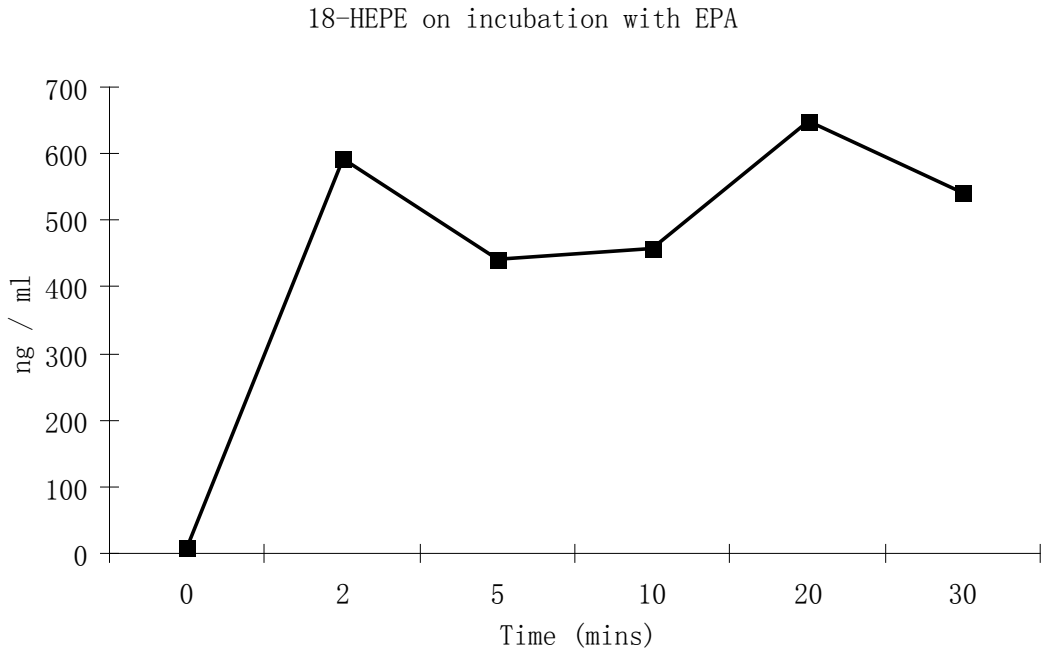
18-HEPE on incubation with EPA
700
600
500
400
300
200
100
0
ng / ml
0
2
5
10
20
30
Time (mins)

**Figure 4.11 (K)**

**Figure 4.12: DHA metabolites generated by HBMEC**

HBMECs were incubated in the absence or presence of 0.1 mM DHA in complete cell culture medium for up to 30 minutes and DHA metabolites determined by mass spectrometry analysis.

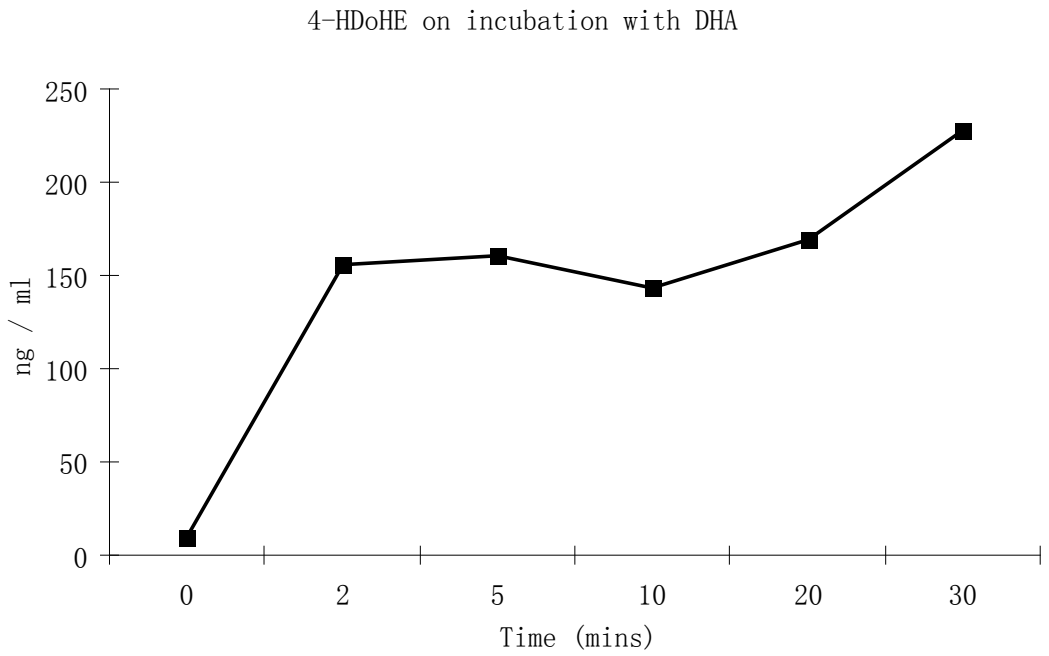


**Figure 4.12 (A)**

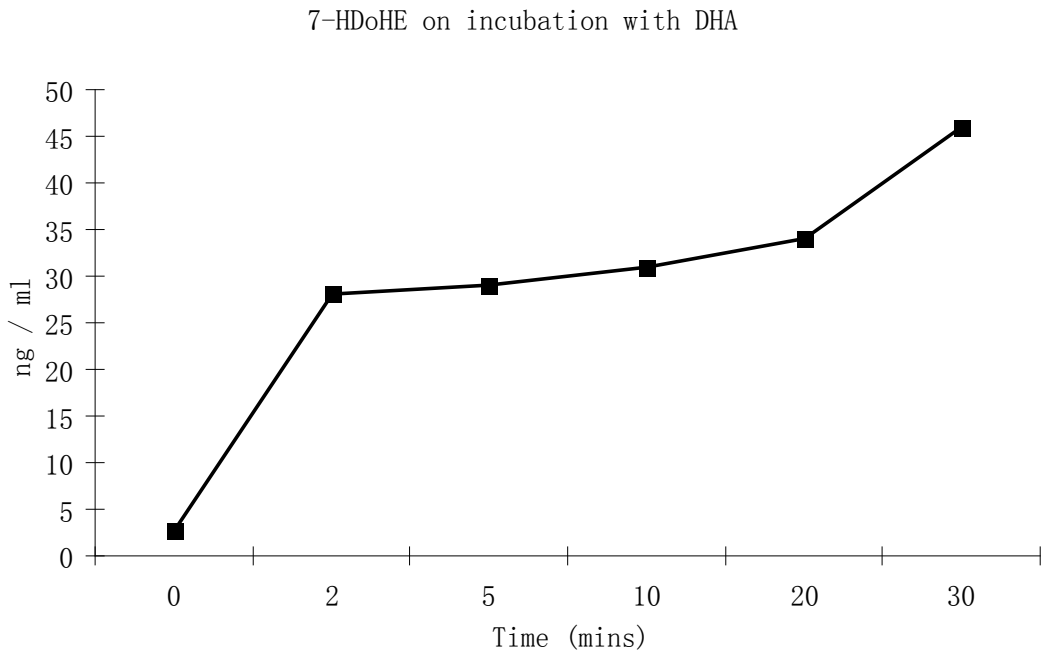


**Figure 4.12 (B)**

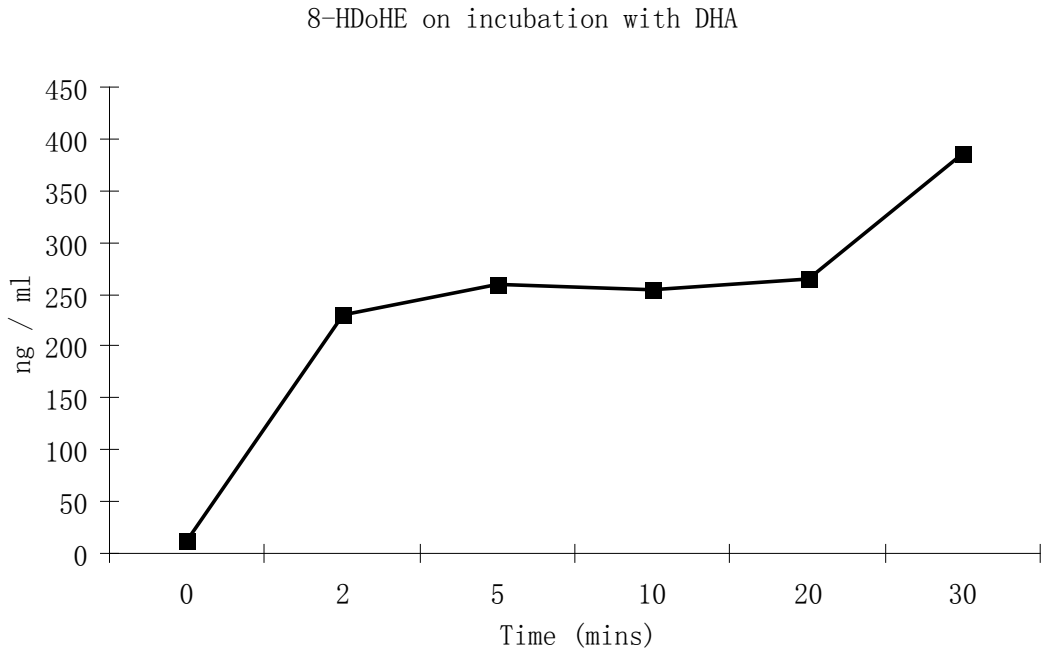


**Figure 4.12 (C)**

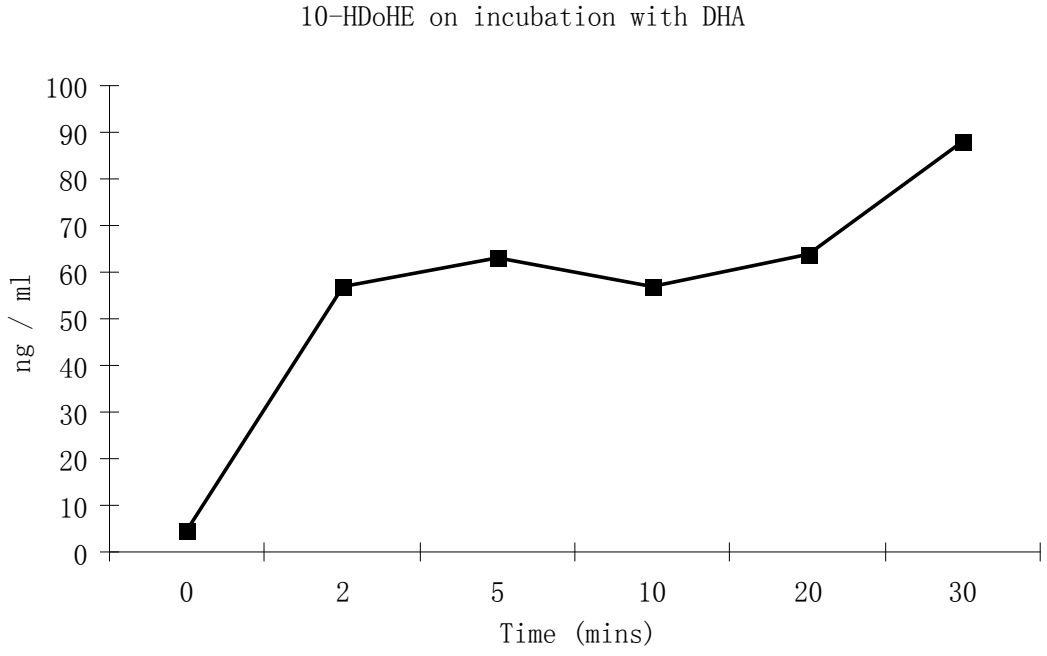


**Figure 4.12 (D)**

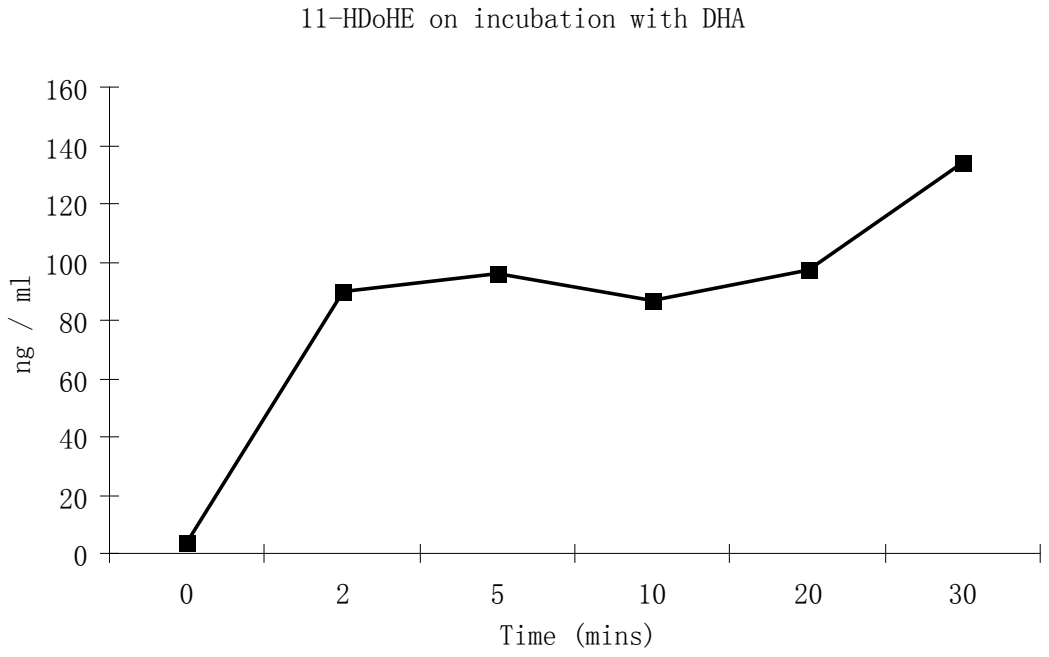


**Figure 4.12 (E)**

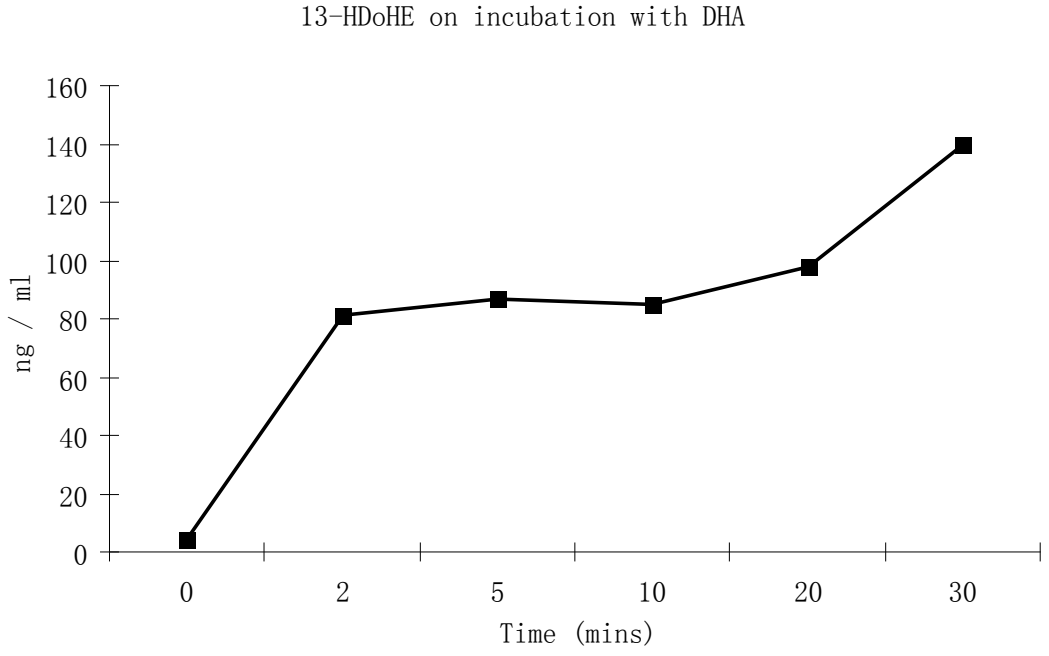


**Figure 4.12 (F)**

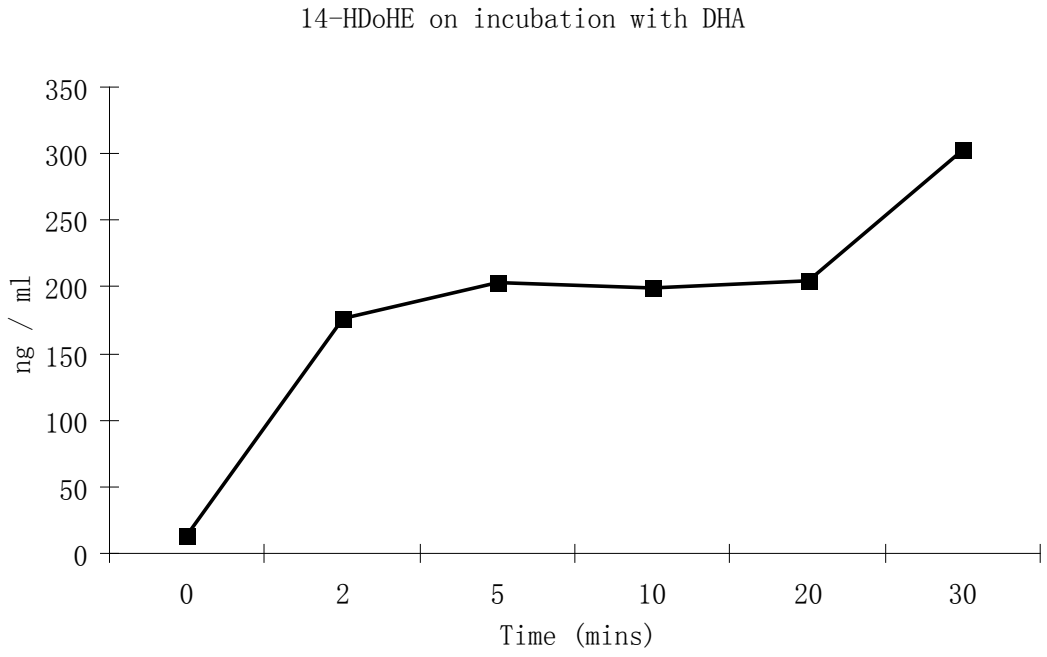


**Figure 4.12 (G)**

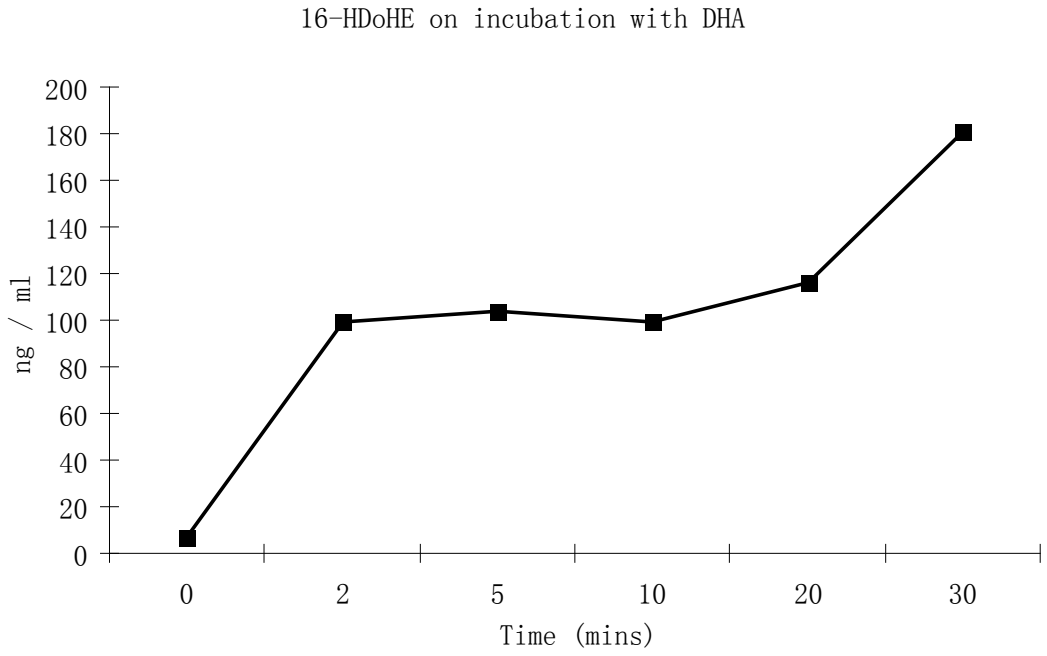


**Figure 4.12 (H)**

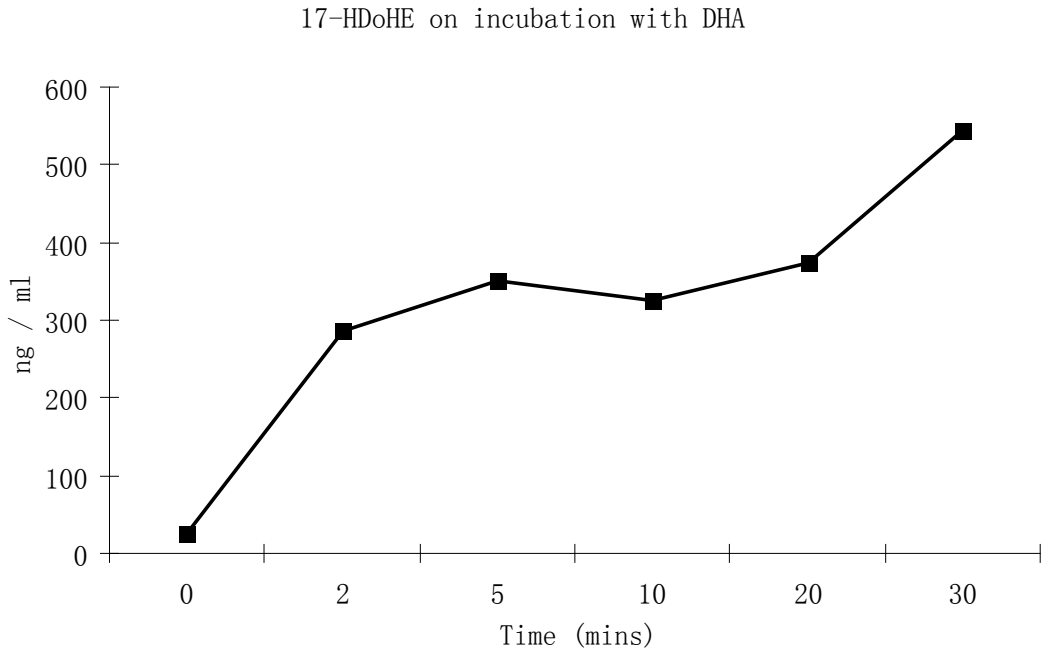


**Figure 4.12 (I)**

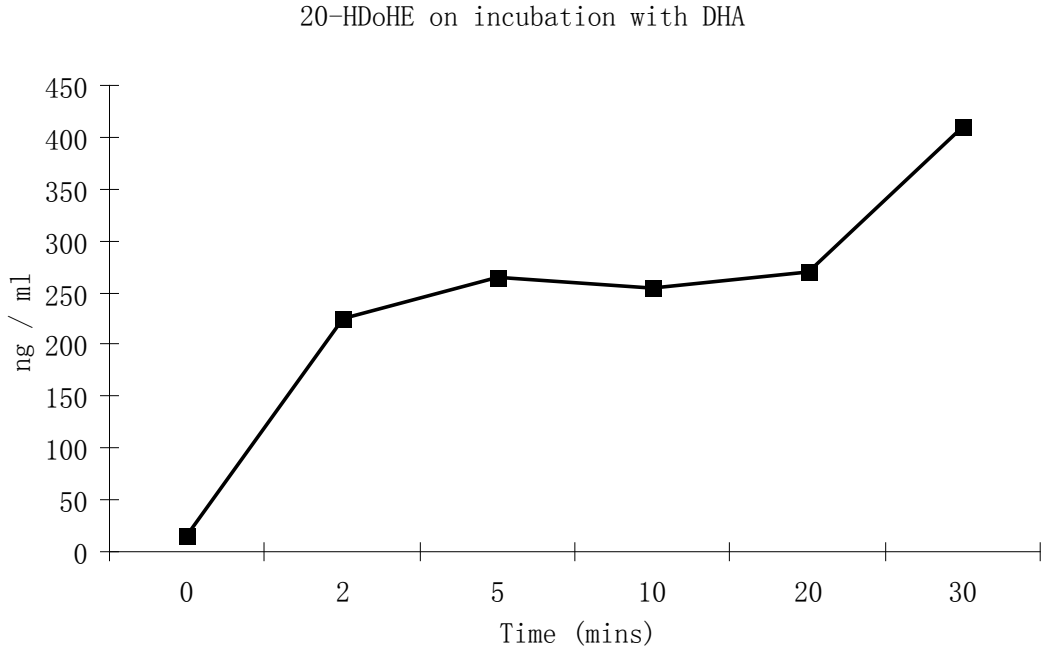


**Figure 4.12 (J)**

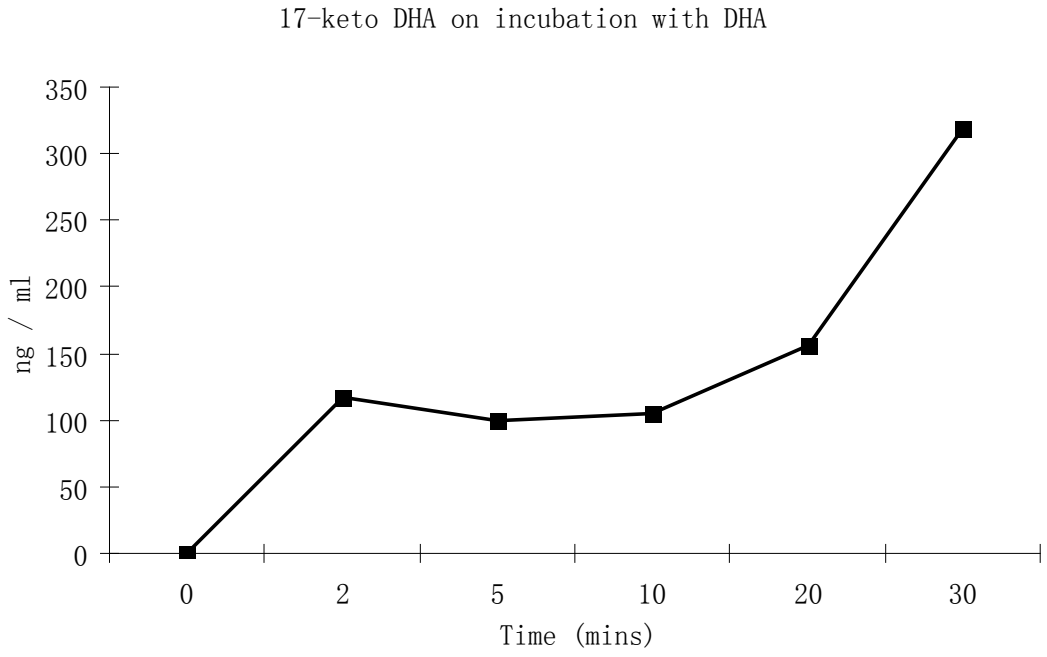


**Figure 4.12 (K)**

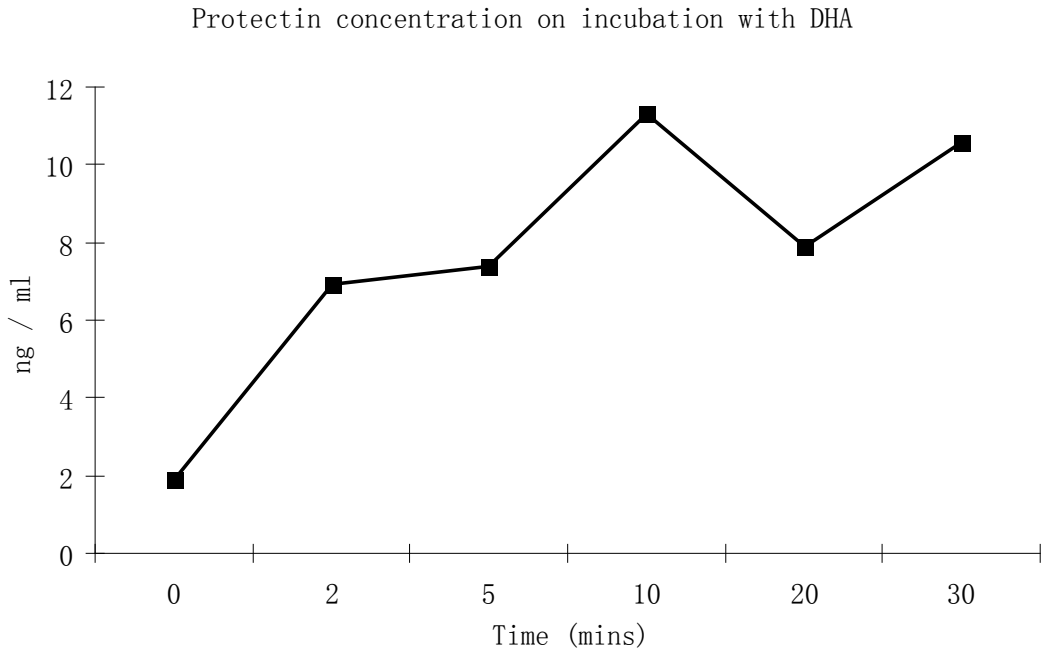


**Figure 4.12 (L)**

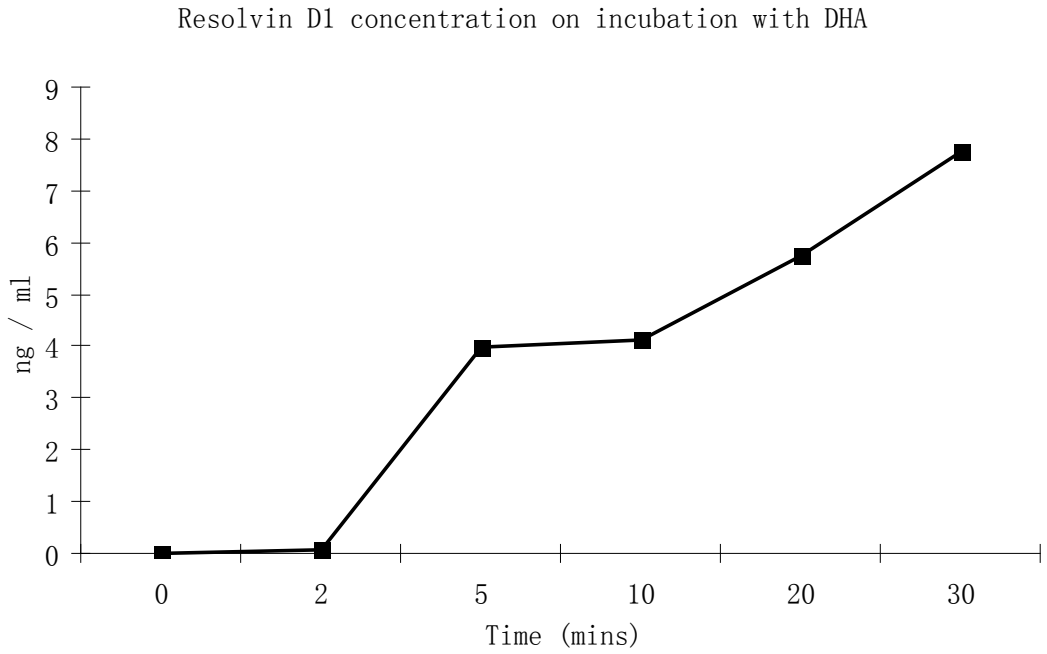


**Figure 4.12 (M)**

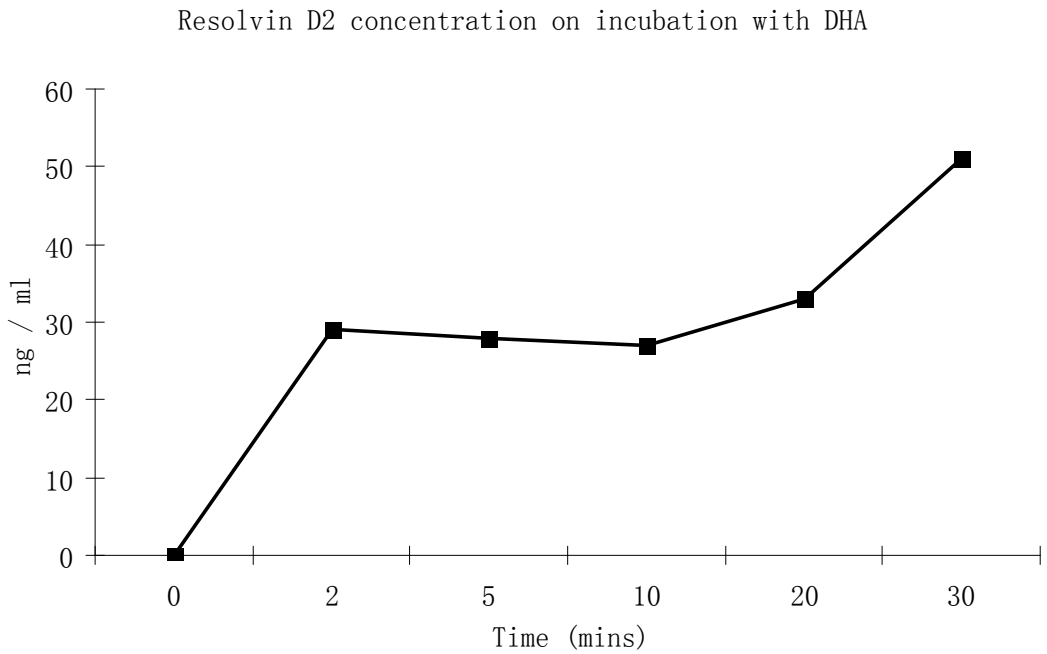


**Figure 4.12 (N)**

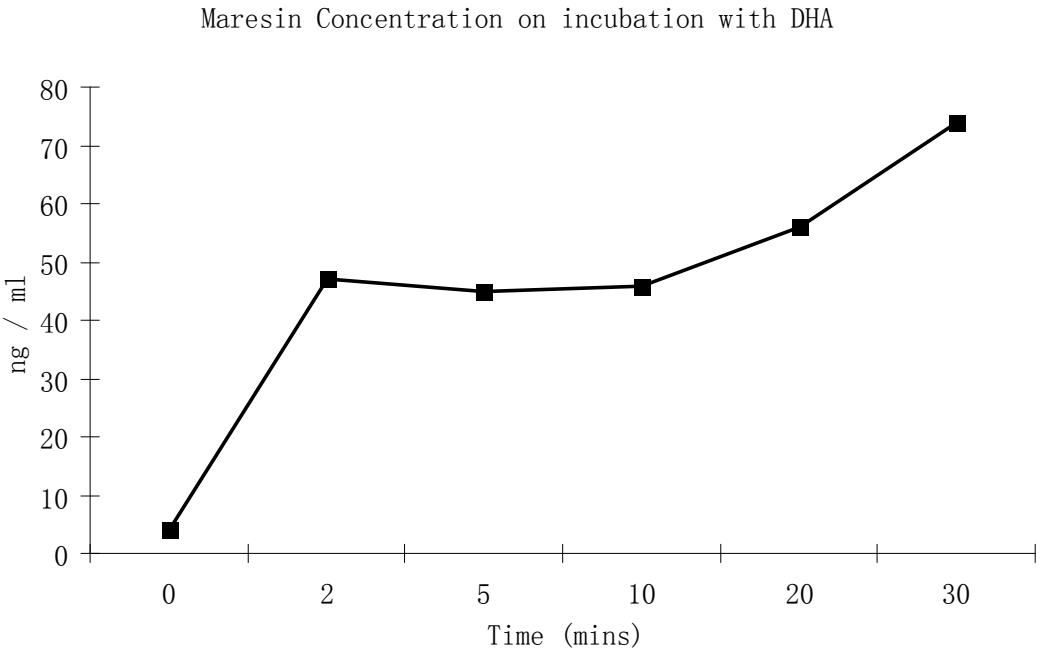
Maresin Concentration on incubation with DHA
80
70
60
50
40
30
20
10
0
ng / ml
0
2
5
10
20
30
Time (mins)

**Figure 4.12 (O)**

**Table 4.1: Metabolite profile on incubation of HBMEC with 0.1 mM arachidonic acid**

| Metabolite | Baseline | 2 min | 5 min | 10 min | 30 min |
|---|---|---|---|---|---|
| **$TXB_2$** | 5.2 | 7.6 | 5.9 | 6.3 | 8.1 |
| **$PGF_{2\alpha}$** | 1.2 | 1.8 | 2.1 | 4.1 | 1.5 |
| **$PGE_2$** | 0.4 | 14.9 | 47 | 33.3 | 12.5 |
| **$PGD_2$** | 1.6 | 59 | 31 | 62 | 53 |
| **$LTB_4$** | 16 | 10.5 | 9 | 7.8 | 7.7 |
| **5-HETE** | 160 | 300 | 475 | 580 | 328 |
| **8-HETE** | 81 | 61 | 119 | 98 | 81 |
| **9-HETE** | 96 | 120 | 179 | 196 | 162 |
| **11-HETE** | 84 | 71 | 125 | 117 | 75 |
| **12-HETE** | 59 | 45 | 84 | 72 | 54 |
| **15-HETE** | 47 | 50 | 92 | 74 | 51 |
| **$LxA_4$** | 6.4 | 87 | 90 | 110 | 88 |

- All results expressed in nanograms per milliliter (ng/ml)

**Table 4.2: Gene expression profile on incubation of HBMEC with 0.1 mM arachidonic acid.**

| Gene | 2 mins | 5 mins | 10 mins | 20 mins | 30 mins |
|---|---|---|---|---|---|
| **EP1** | 0.9 ±0.17 | 0.9 ±0.17 | 0.6 ±0.12 | **0.7*** ±0.06 | 0.7 ±0.17 |
| **EP2** | 1.1 ±0.17 | 0.7 ±0.12 | 0.7 ±0.17 | 0.7 ±0.17 | 0.6 ±0.12 |
| **EP3** | 0.9 ±0.06 | 0.9 ±0.06 | 0.6 ±0.06 | 0.7 ±0.06 | **0.5*** ±0.06 |
| **EP4** | 1.4 ±0.35 | 1.9 ±0.53 | 1.1 ±0.29 | 1.5 ±0.35 | 2 ±0.65 |
| **mPGES** | 1.1 ±0.12 | 1 ±0.17 | 0.9 ±0.06 | 0.9 ±0.12 | 0.7 ±0 |
| **cPGES** | 0.9 ±0.06 | 1.1 ±0.06 | 1.2 ±0.06 | 1.2 ±0.12 | 1 ±0.06 |
| **COX-1** | 0.9 ±0.06 | **0.7*** ±0.12 | **0.7*** ±0 | **0.6*** ±0 | **0.5*** ±0.06 |
| **COX-2** | 1.1 ±0.12 | 1 ±0.06 | 1.1 ±0.06 | 1.2 ±0.12 | 1.1 ±0.12 |
| **5-LOX** | 0.9 ±0.24 | 0.8 ±0.09 | 0.7 ±0.12 | 0.5 ±0.12 | 0.5 ±0.12 |
| **12-LOX** | **0.8*** ±0.06 | **0.7*** ±0 | **0.6*** ±0 | **0.5*** ±0.04 | **0.4*** ±0.04 |
| **$PGD_2R$** | 0.8 ±0.35 | 0.5 ±0.29 | 0.5 ±0.29 | 0.5 ±0.29 | **0.2*** ±0.12 |
| **$LxA_4R$** | 0.8 ±0.12 | **0.7*** ±0.06 | **0.6*** ±0.06 | **0.6*** ±0.06 | **0.4*** ±0.06 |

| | | | | | |
|---|---|---|---|---|---|
| **CysLTR** | 0.7 ±0.24 | **0.5*** ±0.06 | **0.5*** ±0.04 | **0.3*** ±0.06 | **0.2*** ±0.06 |
| **PGT** | 0.8 ±0.06 | 0.8 ±0.06 | **0.7*** ±0.06 | **0.7*** ±0.12 | **0.6*** ±0.06 |
| **MRP4** | 1.1 ±0.12 | 1 ±0.12 | 0.9 ±0.12 | 0.9 ±0.17 | 0.8 ±0.06 |
| **PGDH** | **0.6*** ±0.04 | **0.5*** ±0.04 | **0.5*** ±0.06 | **0.5*** ±0.04 | **0.3*** ±0.04 |
| **Claudin-1** | **0.4*** ±0.06 | **0.4*** ±0.06 | **0.4*** ±0.06 | **0.3*** ±0.06 | **0.2*** ±0.06 |
| **Claudin-3** | 1.9 ±0.41 | 1.5 ±0.17 | 1.5 ±0.35 | 2.6 ±0.76 | 1.8 ±0.59 |
| **Claudin-5** | 1.4 ±0.41 | 1.1 ±0.17 | 0.8 ±0.17 | 1.8 ±0.35 | 1.1 ±0.12 |
| **Claudin-12** | 1.1 ±0.24 | 0.9 ±0.12 | 0.7 ±0 | 1 ±0.17 | 0.6 ±0 |
| **Occludin** | 1.2 ±0.17 | 1.3 ±0.17 | 1.2 ±0.12 | 1.7 ±0.24 | 1.6 ±0.53 |
| **JAM-1** | 1.6 ±0.24 | 1.3 ±0.12 | 1.3 ±0.17 | 2.5 ±0.82 | 1.3 ±0.29 |
| **JAM-3** | 1.1 ±0.07 | 1 ±0.12 | 0.9 ±0.07 | 1.2 ±0.06 | 1 ±0.09 |
| **ZO-1** | 1.3 ±0.07 | 1.1 ±0.04 | 1 ±0.07 | 1.3 ±0.09 | 1.1 ±0.04 |
| **ZO-2** | 1.2 ±0.04 | 1 ±0.04 | 1 ±0.06 | 1.2 ±0.04 | 0.9 ±0.07 |
| **ZO-3** | **0.5*** ±0.04 | **0.5*** ±0.04 | **0.4*** ±0.04 | **0.3*** ±0.07 | **0.2*** ±0.04 |

| | | | | | |
|---|---|---|---|---|---|
| **CLS-1** | 1.2 ±0.24 | 1 ±0.12 | 0.9 ±0.09 | 1.1 ±0.19 | 1 ±0.07 |
| **GLUT-1** | 1 ±0.07 | 0.8 ±0.04 | 0.8 ±0.07 | 1 ±0.09 | 0.7 ±0.07 |
| **PPAR-γ** | 1 ±0.12 | 0.7 ±0.07 | 0.7 ±0.04 | 0.7 ±0.14 | **0.4*** ±0.07 |
| **MMP-2** | 1.1 ±0.15 | 1.2 ±0.29 | 0.9 ±0.19 | 1 ±0.09 | 0.9 ±0.15 |
| **MMP-3** | 0.8 ±0.07 | 0.8 ±0.06 | 0.7 ±0.08 | 0.8 ±0.24 | 0.6 ±0.24 |
| **MMP-9** | 0.8 ±0.08 | 0.8 ±0.18 | **0.5*** ±0.07 | **0.4*** ±0.04 | **0.25*** ±0.03 |

- Data represents fold-change in gene expression compared to baseline levels without incubation with AA.
- Data represents mean of 3 different experiments ± SE.
- Figures highlighted in bold with an asterisk represent statistically significant changes in gene expression ($p<0.05$).

**Table 4.3: Metabolite profile on incubation of HBMEC with EPA**

| Metabolite | Baseline | 2 min | 5 min | 10 min | 20 min | 30 min |
|---|---|---|---|---|---|---|
| **$TxB_3$** | 0.02 | 1.54 | 1.48 | 1.38 | 1.39 | 4.65 |
| **$PGF_{3\alpha}$** | 0.03 | 1.59 | 1.50 | 1.43 | 1.52 | 1.87 |
| **$PGE_3$** | 1.08 | 256 | 192 | 203 | 172 | 170 |
| **$PGD_3$** | 0 | 0 | 235 | 258 | 146 | 750 |
| **5-HEPE** | 9.01 | 479 | 340 | 356 | 532 | 404 |
| **8-HEPE** | 6.71 | 1053 | 836 | 813 | 532 | 871 |
| **9-HEPE** | 3.69 | 311 | 254 | 228 | 397 | 271 |
| **11-HEPE** | 2.97 | 180 | 143 | 142 | 194 | 163 |
| **12-HEPE** | 3.4 | 231 | 188 | 180 | 252 | 193 |
| **15-HEPE** | 4.09 | 294 | 222 | 209 | 289 | 274 |
| **18-HEPE** | 6.95 | 591 | 441 | 457 | 649 | 540 |

- All results expressed in nanograms per milliliter (ng/ml)

**Table 4.4: Metabolite profile on incubation of HBMEC with DHA**

| Metabolite | Baseline | 2 min | 5 min | 10 min | 20 min | 30 min |
|---|---|---|---|---|---|---|
| **4-HDoHE** | 9.46 | 156 | 161 | 143 | 169 | 228 |
| **7-HDoHE** | 2.64 | 28 | 29 | 31 | 34 | 46 |
| **8-HDoHE** | 12 | 231 | 259 | 255 | 264 | 386 |
| **10-HDoHE** | 4.51 | 57 | 63 | 57 | 64 | 88 |
| **11-HDoHE** | 3.52 | 90 | 96 | 87 | 97 | 134 |
| **13-HDoHE** | 4.5 | 81 | 87 | 85 | 98 | 140 |
| **14-HDoHE** | 13 | 176 | 203 | 199 | 205 | 303 |
| **16-HDoHE** | 7.06 | 99 | 104 | 99 | 116 | 181 |
| **17-HDoHE** | 25 | 286 | 351 | 326 | 375 | 544 |
| **20-HDoHE** | 16 | 225 | 265 | 255 | 270 | 410 |
| **17-keto DHA** | 0 | 117 | 100 | 105 | 156 | 319 |
| **Protectin** | 1.89 | 6.9 | 7.4 | 11.3 | 7.89 | 10.55 |
| **Resolvin $D_1$** | 0 | 0.07 | 3.99 | 4.13 | 5.76 | 7.77 |
| **Resolvin $D_2$** | 0 | 29 | 28 | 27 | 33 | 51 |
| **Maresin** | 4.11 | 47 | 45 | 46 | 56 | 74 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **9-OXO ODE** | 34 | 61 | 42 | 37 | 68 | 166 |
| **13-OXO ODE** | 16 | 38 | 27 | 23 | 40 | 104 |

- All results expressed in nanograms per milliliter (ng/ml)

# 5. DISCUSSION

The objective of the present study was to examine the effect of arachidonic acid on the permeability and behavior of the HBMEC. HBMEC plated on Transwell® inserts were used in the present study as an in vitro model of the blood-brain barrier. Several previous studies have established the utility of this model of the BBB. Some of these studies have used additional components along with the HBMEC such as astrocyte co-culture (Cecchelli *et al*. 1999) or astrocyte-conditioned medium (Rubin *et al*. 1991). Yet others have established models using a primary culture of the HBMEC as the sole component (Miller *et al*. 1992; Mitchell *et al*. 2009; Mitchell *et al*. 2011; Trickler *et al*. 2005; de Vries *et al*. 1996).

Previously, it was demonstrated in our laboratory that the transport of various fatty acids across confluent layers of HBMEC was, in part, mediated by fatty acid transport proteins (Mitchell *et al*. 2009, 2011). However, the role that fatty acids themselves play in HBMEC permeability was unknown.

We first incubated confluent monolayers of HBMEC with 0.1 mM AA and observed that there was increased paracellular flux of FDX through the incubation period. Thus, we observed that incubation with AA appears to increase the permeability of the HBMEC monolayers. This is in agreement with previous studies that have

documented permeability-enhancing and neurotoxic effects of AA (Chan *et al*. 1983; Chan *et al*. 1985; Katsuki and Okuda 1995). Our observations may explain why there is rapid influx of AA into the brain upon plasma infusion with AA (Rapoport *et al*. 2001; Duncan and Bazinet 2010).

AA is a precursor for the formation of various bioactive molecules including prostaglandins and leukotrienes. Several studies have shown that the increase in BBB permeability is correlated with the formation of $PGE_2$ (Jaworowicz *et al*. 1998; Stanimirovic and Satoh 2000; de Vries *et al*. 1996; Trickler *et al*. 2005; Deli *et al*. 2005; Mark *et al*. 2001). Jiang *et al*. have shown that prostaglandin EP2 receptor is responsible for mediating the neuroinflammatory and neurodegenerative effects of $PGE_2$ in mice models of status epilepticus (Jiang *et al*. 2013).

We postulated that the increase in permeability caused by AA could be due to the production of $PGE_2$. Also, if that were true, the NSAIDs would be able to completely or partially restore the permeability of the HBMEC. However, in our experiments, incubation of HBMEC with NSAIDs did not alter the AA-mediated increased permeability of the HBMEC monolayer. Next, we incubated the HBMEC with AA and exogenously added $PGE_2$. Surprisingly, we observed that adding $PGE_2$ led to a significant decrease in permeability and ‘tightening up’ of the

HBMEC monolayer. We observed a 20% and 45% decrease in the transport of arachidonic acid from apical to basolateral compartment at 20 minutes and 30 minutes, respectively, using a concentration of 100 ng/ml of $PGE_2$.

The permeability studies were repeated using a 10-fold lower concentration of AA (10 μM). On measuring the FDX flux across the HBMEC monolayer, we noticed a similar increase in permeability as seen with our previous experiments using 0.1 mM AA. Again, adding $PGE_2$ (100 ng/ml) to the inserts led to a significant decrease in permeability of the HBMEC monolayer. $PGE_2$ reduced the permeability of the HBMEC monolayer by 16% at 2 minutes, 26% at 5 minutes, 40% at 10 minutes, 52% at 20 minutes and 54% at 30 minutes. The results were statistically significant ($p < 0.0001$).

The permeability studies were repeated using lower concentrations of $PGE_2$ (50, 25 and 12.5 ng/ml). At each of these concentrations, we found a significant decrease in the transport of AA from the apical to the basolateral side of the HBMEC. We found a 14-24% reduction in the transport of AA to the basolateral side of the HBMEC monolayer at 10 minutes, a 23-30% decrease at 20 minutes and a 33% decrease at 30 minutes. The decreases were statistically significant at these time points of 10, 20 and 30 minutes. Thus, we concluded that exogenously added $PGE_2$ prevents AA-mediated permeability

increases of the HBMEC monolayer, to a similar extent, across a concentration range of 12.5 through to 100 ng/ml.

A few studies have documented that $PGE_2$, in fact, has a protective role in the microvessels of the CNS and that it prevents permeability increases. Studies by Farmer *et al*. have shown that permeability increase caused by bradykinin can be prevented or attenuated by exogenously added $PGE_2$ and iloprost, a prostacyclin analog (Farmer *et al*. 2001). In their studies, COX-inhibitor drugs potentiated the permeability increases caused by bradykinin, thus suggesting an inhibitory role of $PGE_2$ in increasing endothelial cell permeability.

Studies by Esaki *et al*. have shown that $PGE_2$, acting via EP4 receptors, inhibited the increase in BBB permeability in a mouse model of experimental autoimmune encephalomyelitis (EAE) (Esaki *et al*. 2010). Studies by Ahmad *et al*. have shown that $PGE_2$, acting via EP2 receptors, has neuroprotective properties and limits ischemic damage in mice stroke models (Ahmad *et al*. 2010). It has been postulated in these studies (Farmer *et al*. 2001; Esaki *et al*. 2010) that engagement of EP2 and EP4 receptors by $PGE_2$ leads to an increase in cAMP levels. This cAMP accumulation has been shown to potentiate cadherin-mediated cell-cell contact and enhance endothelial barrier function. Thus, $PGE_2$ may promote BBB integrity via direct action on endothelial cells (Esaki *et al*. 2010).

Easton and Fraser have shown that the permeability increase caused by AA in pial microvessels of rats was effectively blocked by a combination of indomethacin (COX inhibitor) and nordihyroguariaretic acid (LOX inhibitor) but not singly by either agent (Easton and Fraser 1998). In the same study, AA-mediated permeability increase was blocked by superoxide dismutase and catalase. The authors concluded that free radicals generated by either COX or LOX pathways were responsible for the permeability response to AA. Othman *et al*. have shown in mice models of diabetic retinopathy that 12-HETE and 15-HETE, products of the lipoxygenase pathway, are responsible for increasing the permeability of retinal endothelial cell barrier via NADPH oxidase-dependent mechanism (Othman *et al*. 2013).

Thus, from our present study, it seems likely that AA-mediated increase in permeability of the HBMEC is mediated by some metabolite of the LOX or epoxygenase pathway, but not by $PGE_2$ or other metabolites of the COX pathway and that $PGE_2$ may, in fact, have a protective effect on the tight junctions of the BBB.

To further examine the role of transport proteins in the cellular uptake of AA, we used HBMEC monolayers in which FATP-1 and CD36 were knocked down using RNAi and incubated them with AA. We did not observe any difference in the transport of radiolabeled-AA from the apical to basolateral side of the monolayer. This is in contrast to

previous studies in our laboratory that have shown that transport proteins like FATP-1 and CD36 play an important role in the cellular uptake of long chain fatty acids like oleic acid (Mitchell *et al*. 2009; 2011). This lack of inhibition by fatty acid transport protein knock down could have been due to altered transcellular or paracellular passage of AA. Incubation of HBMEC with AA appeared to increase permeability of the HBMEC monolayer as indicated by an increased flux of FDX into the basolateral medium. Thus, AA appeared to increase the paracellular permeability across HBMEC.

Phloretin, a type of natural phenol, is a non specific inhibitor of fatty acid transport proteins. Incubation of phloretin-treated HBMEC led to a similar degree of permeability increase when compared with HBMEC without phloretin exposure. These studies were performed as an extension of the siRNA knock down studies and confirmed the fact that fatty acid transport proteins are not essential for the transport of AA across the HBMEC monolayer.

Several studies have demonstrated that microvessel endothelial cells from various organs have the capacity to produce a range of eicosanoids, notably, $PGE_2$, $PGD_2$, $PGI_2$ and $PGF_{2\alpha}$. However, in most of these studies, the endothelial cells were stimulated with the calcium ionophore A23187 in addition to exogenously added AA (Denning *et al*. 1982; Eldor *et al*. 1983; Gerritsen *et al*. 1987). In a

study by Baker *et al*., the endothelial cells exposed to plasma from preeclamptic women showed increased production of prostaglandins (Baker *et al*. 1996). In a study by Trickler *et al*., bovine brain microvessel endothelial cells (BBMEC) exposed to TNF-α released large amounts of $PGE_2$ over a 12-hour period (Trickler *et al*. 2005).

In our study, we incubated the HBMEC with AA or EPA or DHA for up to 30 minutes and assayed the cell culture supernatant at various time points. Our mass spectroscopic analysis showed that the HBMEC are capable of producing a variety of eicosanoids and docosanoids on unstimulated incubation with AA or EPA or DHA and as early as within two minutes of incubation.

Incubation of HBMEC with AA led to an increased production of both pro- and anti-inflammatory metabolites. There was a 50-fold increase in the release of $PGE_2$ at 5 minutes of incubation. $PGE_2$ is known to exert both pro- and anti-inflammatory effects, as discussed earlier. Similarly, there was a marked increase in the release of $PGD_2$, 5-HETE, 8-HETE, 9-HETE, 12-HETE and 15-HETE, all of which are known to exert pro-inflammatory effects. However, we also observed a 20-fold increase in the release of $LxA_4$, an anti-inflammatory molecule. At the same time, we also observed a 2-fold drop in the release of $LTB_4$. Thus, it is apparent that incubation with AA leads to increased production and/or release of pro-inflammatory as well as anti-inflammatory

molecules, which probably balance or oppose each other's effects. This could be important in maintaining homeostasis in various tissues of the body as they are exposed to dietary arachidonic acid.

In similar experiments, incubation of HBMEC with EPA or DHA led to the production and/or release of exponential amounts of a number of metabolites produced primarily by the lipoxygenase or CYP450 ω -hydroxylase pathways. Additionally, incubation of HBMEC with EPA also led to the production of large amounts of prostaglandins of the '3' (trienoic) series.

Previous studies have shown that DHA is converted to its vasodilator metabolite, 17S-HDoHE in endothelial cells (Li *et al*. 2011). Hong *et al*. have shown that DHA is a precursor in the formation of several bioactive molecules in human blood cells and glial cells (Hong *et al*. 2003). However, in their experiments, the cells were exposed to stimulants like zymosan A or the calcium ionophore, A23187 to facilitate the release of DHA metabolites. Our studies have shown that the HBMEC are capable of producing and/or releasing a vast variety of metabolites on unstimulated incubation with EPA or DHA. These n-3 PUFA metabolites have been shown to have several biological effects like inhibition of inflammation and platelet aggregation, mediation of vasodilation, anti-arrhythmic effects and lowering of triglyceride levels (Adkins and Kelley 2010).

We analyzed the expression profile of several genes involved in the production and action of eicosanoids and those involved in the formation and maintenance of tight junctions between the HBMEC. We observed that the mRNA levels of several genes were significantly decreased on incubation of HBMEC with AA. These down-regulated genes included the genes for EP1, EP3, COX-1, PGT, $PGD_2$ receptor, $LxA_4$ receptor, cysteinyl LT receptor, 12-LOX, PPAR-$\gamma$, ZO-3, claudin-1, MMP-9 and prostaglandin dehydrogenase. On the other hand, it was observed that the expression of a few genes was up-regulated at certain time points on incubation with AA, though the level of up-regulation did not reach statistical significance. The genes were those encoding EP4, JAM-1 and claudin-3. The expression of yet other genes remained unchanged in response to AA incubation. These were the genes for EP2, COX-2, mPGES-1, cPGES, occludin, claudin-5, claudin-12, ZO-1, ZO-2, JAM-3, $MRP_4$, 5-LOX, cardiolipin synthase, GLUT-1, MMP-2 and MMP-3.

Our studies are consistent with previous studies such as that by Stuhlmeier *et al*. in which AA inhibited the cytokine-induced up-regulation of several genes involved in endothelial cell inflammation (Stuhlmeier *et al.* 1996). Since, in our study, we observed the gene expression changes with a short incubation time of 30 minutes, it seems likely that the majority of mRNA changes would

reflect changes in mRNA transcription that may not immediately correlate with changes in protein expression. We plan to carry further studies to examine the protein-level changes of these biomolecules by performing Western blots.

As mentioned previously, $PGE_2$ is known to exert its effects by binding to G protein- coupled seven- transmembrane spanning receptors located in the plasma membrane as well as the nuclear membrane (Funk 2001; Bhattacharya *et al*. 1998). Four subtypes of $PGE_2$ receptors exist: EP1, EP2, EP3 and EP4 (Dubois et al. 1998; Hata and Breyer 2004; McCoy *et al*. 2002). HBMEC expressed all four subtypes of $PGE_2$ receptor. In addition, in our studies, the mRNA for EP1 and EP3 appeared to be significantly down-regulated on incubating the HBMEC with AA for up to 30 minutes. This is consistent with previous studies which have shown a decrease in plasma membrane EP receptors in response to sustained, high levels of prostaglandins (Bhattacharya *et al*. 1998). As mentioned earlier, our mass spectrometric studies have also established that there is a marked increase in the production and/or release of $PGE_2$ on incubating the HBMEC with arachidonic acid. Thus, it seems possible that high levels of $PGE_2$ inhibit the expression of EP1 and EP3 by a negative feedback. Some studies have also described sensitization of the $PGE_2$ receptor as means to down- regulate the downstream actions of sustained,

elevated $PGE_2$ (Negishi *et al*. 1995). In our studies, we did notice a trend toward down- regulation of the EP2 receptor and up- regulation of the EP4 receptor, though the results were not statistically significant. It is also worthwhile to note that $PGE_2$ may have opposing actions depending on its interaction with different EP receptor subtypes that are coupled to different signal transduction pathways (Li *et al*. 1994; Dumont *et al*. 1998).

In an effort to study the transport of EPA and DHA across the blood-brain barrier, we performed similar permeability studies in Transwell® inserts using FDX as a marker for paracellular passage of macromolecules. We did not notice any significant change in the permeability of the HBMEC monolayer to FDX, suggesting that EPA or DHA did not affect the permeability of the HBMEC.

In the future, we would like to pin-point the metabolite responsible for the AA- mediated increases in permeability of the HBMEC monolayer, and hence, the blood-brain barrier. This would help us design new therapies to increase drug delivery to the brain. We plan to accomplish this by studying the effect of specific metabolites of the lipoxygenase and epoxygenase pathways on permeability of the HBMEC monolayers. Confirmatory studies would be performed using pharmacological inhibitors or receptor blockers of these metabolites. One limitation of our gene expression study is that we have not

examined if the protein changes correlate with the changes in gene expression. We plan to perform western blots for semi- quantitative analysis of these proteins whose gene expression is altered by arachidonic acid. Further, we would also like to delve deeper into the mechanisms of transport of EPA and DHA into the HBMEC.

# 6. CONCLUSIONS

1. Arachidonic acid increases the permeability of the HBMEC monolayers and thus, may modulate the permeability of the blood brain barrier in vivo.
2. Transport of arachidonic acid across the HBMEC does not require participation of fatty acid transport proteins.
3. $PGE_2$ attenuates the AA-mediated increase in permeability of the HBMEC monolayers.
4. The AA-mediated increase in permeability is likely caused by metabolites other than $PGE_2$, possibly metabolites of the lipoxygenase pathway.
5. Arachidonic acid alters the expression of several genes involved in prostaglandin production, metabolism and transport and in tight junction formation and maintenance, possibly at the level of transcription.
6. Incubation of HBMEC monolayers with EPA or DHA does not alter their permeability.
7. Exposure of HBMEC to exogenous AA, EPA or DHA leads to an exponential increase in the production and/or release of numerous bioactive metabolites of the respective fatty acids.

# 7. REFERENCES


Abbott N.J., Ronnback L. and Hansson E. (2006) **Astrocyte-endothelial interactions at the blood-brain barrier**. *Nature Reviews Neuroscience*. **7**, 41- 53.

Adkins Y. and Kelley D.S. (2010) **Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids**. *J. Nutritional Biochem*. **21**, 781-792.

Ahmad M., Saleem S., Shah Z., Maruyama T., Narumiya S. and Dore S. (2010) **The $PGE_2$ EP2 receptor and its selective activation are beneficial against ischemic stroke**. *Experimental and Translational Stroke Medicine*. **2**, 12.

Arnold C., Konkel A., Fischer R. and Schunck W.-H. (2010) **Cytochrome P450-dependent metabolism of ω-6 and ω-3 long-chain polyunsaturated fatty acids**. *Pharmacological Reports*. **62**, 536-547.

Back M., Dahlen S.-E., Drazen J.M., Evans J.F., Serhan C.N., Shimuzu T., Yokomizo T. and Rovati G.E. (2011) **International union of basic and clinical pharmacology. LXXXIV: Leukotriene receptor**

**nomenclature, distribution and pathophysiological functions**. *Pharmacol. Rev*. **63**, 539-584.

Baker P.N., Davidge S.T., Barankiewicz J. and Roberts J.M. (1996) **Plasma of preeclamptic women stimulates and then inhibits endothelial prostacyclin**. *Hypertension*. **27**, 56-61.

Balabanov R. and Dore-Duffy, P. (1998) **Role of the CNS microvascular pericyte in the blood-brain barrier**. *J. Neurosci. Res.* **53**, 637-644

Ballabh P., Braun A. and Nedergaard M. (2004) **The blood-brain barrier: an overview: Structure, regulation and clinical implications**. *Neurobiol. Dis*. **16**, 1-13

Bannenberg G. and Serhan C.N. (2010) **Specialized pro-resolving lipid mediators in the inflammatory response: An update**. *Biochim. Biophys. Acta*. **1801**, 1260-1273.

Bazzoni G. (2003) **The JAM family of Junctional adhesion molecules**. *Curr. Opin. Cell Biol*. **15**, 525-530

Bhattacharya M., Peri K.G., Almazan G., Ribeiro-da-Silva A., Shichi H., Durocher Y., Abramovitz M., Hou X., Varma D.R. and Chemtob S. (1998) **Nuclear localization of prostaglandin E2 receptors**. *Proc. Natl. Acad. Sci. USA*. **95**, 15792-15797

Bhattacharya M., Peri K., Ribeiro-da-Silva A., Almazan G., Shichi H., Hou X., Varma D.R. and Chemtob S. (1999) **Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope**. *J. Biol. Chem*. **274**, 15719-15724.

Bos C.L., Richel D.J., Ritsema T., Peppelenbosch M.P. and Versteeg H.H. (2004) **Prostanoids and prostanoid receptors in signal transduction**. *The International J. Biochem. Cell Biol.* **36**, 1187-1205

Bosetti F. (2007) **Arachidonic acid metabolism in brain physiology and pathology: lessons from genetically altered mouse models**. *J. Neurochem*. **102**, 577-586.

Brash A.R. (2001) **Arachidonic acid as a bioactive molecule**. *J. Clin. Investigation*. **107**, 1339-1345.

Burke P.A., Ling P.R., Forse R.A. and Bistrian B.R. (1999) **Conditionally essential fatty acid deficiencies in end- stage liver disease**. *Nutrition*. **15**, 302-304.

Cecchelli R., Dehouck B., Descamps L., Fenart L., Buee-Scherrer V., Duhem C., Lundquist S., Rentfel M., Torpier G. and Dehouck M.P. (1999) **In vitro model for evaluating drug transport across the blood-brain barrier**. *Adv. Drug Deliver. Rev.* **36**, 165-178.

Chan B.S., Satriano J.A., Pucci M and Schuster V.L. (1998) **Mechanism of prostaglandin $E_2$ transport across the plasma membrane of HeLa cells and *Xenopus* oocytes expressing the prostaglandin transporter "PGT"**. *J. Biol. Chem*. **273**, 6689-6697.

Chan P.H., Fishman R.A., Caronna J., Schmidley J.W., Prioleau G. and Lee J. (1983) **Induction of brain edema following intracerebral injection of arachidonic acid**. *Ann Neurol*. **13**, 625-632

Chan P.H., Fishman R.A., Longar S., Chen S. and Yu A. (1985) **Cellular and molecular effects of polyunsaturated fatty acids in brain ischemia and injury**. *Prog. Brain Res*. **63**, 227-236.

Chiu P.-S. and Lai S.-C. (2013) **Matrix metalloproteinase-9 leads to claudin-5 degradation via the NF-κB pathway in BALB/c mice with eosinophilic meningoencephalitis caused by *Angiostrongylus cantonensis***. *PLOS One*. **8**, e53370.

Dallas S., Miller, D.S. and Bendayan R. (2006) **Multidrug resistance-associated proteins: Expression and function in the central nervous system**. *Pharmacol. Rev*. **58**, 140-161.

Das U.N. (2010) **Lipoxins, resolvins, protectins, maresins and nitrolipids: Connecting lipids, inflammation and cardiovascular disease risk**. *Curr. Cardio. Risk Rep*. **4**, 24-31.

Deems, R., Buczynski, M.W., Bowers-Gentry, R., Harkewicz, R., Dennis, E.A. (2007) **Detection and quantitation of eicosanoids via high performance liquid chromatography- electrospray ionization-mass spectrometry**. *Methods Enzymol*. **432**, 59-82.

Deli M.A., Abraham C.S., Kataoka Y. and Niwa M. (2005) **Permeability studies on in vitro blood-brain barrier models: Physiology, pathology and pharmacology**. *Cell. Mol. Neurobiol*. **25**, 59-127.

Denning G.M., Figard P.H. and Spector A.A (1982) **Effect of fatty acid modification on prostaglandin production by cultures 3T3 cells**. *J. Lipid Res*. **23**, 584-596.

Dorovini-Zis, K., Bowman, P.D., Betz, A.L. and Goldstein, G.W. (1987) **Formation of a barrier by brain microvessel endothelial cells in culture**. *Fed. Proc.* **46**, 2521-2522.

Dubois R.N., Abramson S.B., Crofford L., Gupta R.A., Simon L.S., van de Putte L.B.A. and Lipsky P.E. (1998) **Cyclooxygenase in biology and disease**. *FASEB J.* **12**, 1063-1073.

Dumont I., Peri K.G., Hardy P., Hou X., Martinez-Bermudez A.K., Molotchnikoff S., Varma D.R. and Chemtob S. (1998). **$PGE_2$, via EP3 receptors, regulates brain nitric oxide synthase in the perinatal period**. *Am. J. Physiol.* **275**, R1812-R1821.

Duncan R.E. and Bazinet R.P. (2010) **Brain arachidonic acid uptake and turnover: implications for signalling and bipolar disorder**. *Curr. Opin. Clin. Nutr. Metab. Care.* **13**, 130-138.

Easton A.S. and Fraser P.A. (1998) **Arachidonic acid increases cerebral microvascular permeability by free radicals in single pial microvessels of the anaesthetized rat**. *J. Physiol*. **507.2**, 541-547.

Eldor A., Vlodavsky I., Hy-Am E., Atzmon R., Weksler B.B., Raz A. and Fuks Z. (1983) **Cultured endothelial cells increase their capacity to synthesize prostacyclin following the formation of a contact inhibited cell monolayer**. *J. Cell. Physiol*. **114**, 179-183.

Engblom D., Ek M., Saha S., Ericsson-Dahlstrand A., Jakobsson P and Blomqvist A. (2002) **Prostaglandins as inflammatory messengers across the blood-brain barrier**. *J. Mol. Med*. **80**, 5-15.

Esaki Y., Li Y., Sakata D., Yao C., Segi-Nishida E., Matsuoka T., Fukuda K. and Narumiya S. (2010) **Dual roles of $PGE_2$-EP4 signaling in mouse experimental autoimmune encephalomyelitis**. *Proc. Natl. Acad. Sciences USA*. **107**, 12233-12238.

Evans J.F. (2003) **The cysteinyl leukotriene receptors**. *Prostaglandins Leukotrienes Ess. Fatty Acids.* **69**, 117-122.

Farmer P.J., Bernier S.G., Lepage A., Guillemette G., Regoli D. and Sirois P. (2001) **Permeability of endothelial cell monolayers to albumin is increased by bradykinin and inhibited by prostaglandins**. *Am. J. Physiol. Lung Cell. Mol. Physiol*. **280**, L732-L738.

Feldman G.J., Mullin J.M. and Ryan M.P. (2005) **Occludin: Structure, function and regulation**. *Advanced Drug Delivery Rev.* **57**, 883-917.

Flamand N., Mancuso P., Serezani C.H.C. and Brock T.G. (2007) **Leukotrienes: Mediators that have been typecast as villains**. *Cell. Mol. Life Sci*. **64**, 2657-2670.

Funk C.D. (2001) **Prostaglandins and leukotrienes: Advances in eicosanoid biology**. *Science*. **294**, 1871- 1875

Furuse M., Fujita K., Hiiragi T., Fujimoto K. and Tsukita S. (1998) **Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin**. *J. Cell Biol.* **141**, 1539-1550.

Gerritsen M.E., Nganele D.M. and Rodrigues A.M. (1987) **Calcium ionophore (A23187)- and arachidonic acid- stimulated prostaglandin release from microvascular endothelial cells: Effects of calcium antagonists and calmodulin inhibitors**. *J. Pharmacol. Experimental Therapeutics*. **240**, 837-846.

Gloor S.M., Wachtel M., Bolliger M.F., Ishihara H., Landmann R. and Frei K. (2001) **Molecular and cellular permeability control at the blood-brain barrier**. *Brain Res. Rev.* **36**, 258-264.

Gobeil F., Vazquez-Tello A., Marrache A.M., Bhattacharya M., Checchin D., Bkaily G., Lachapelle P., Ribeiro-Da-Silva A. and Chemtob S. (2003) **Nuclear prostaglandin signalling system: biogenesis and actions via heptahelical receptors**. *Can. J. Physiol. Pharmacol.* **81**, 196-204.

Goldstein, G.W. and Betz, A.L. (1983) **Recent advances in understanding brain capillary function**. *Ann Neurol.* **14**, 389-395.

Gross G.J., Falck J.R., Gross E.R., Isbell M., Moore J. and Nithipatikom K. (2005) **Cytochrome P450 and arachidonic acid metabolites: Role in myocardial ischemia/reperfusion injury revisited**. *Cardiovascular Research*. **68**, 18-25.

Gumbleton M. and Audus K.L. (2001) **Progress and limitations in the use of *in vitro* cell cultures to serve as a permeability screen for the blood-brain barrier**. *J. Pharm. Sci.* **90**, 1681-1698.

Haseloff R.F., Blasig I.E., Bauer H.-C. and Bauer H. (2005) **In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells in vivo**. *Cellular Mol. Neurobiol*. **25**, 25-

Hata A.N. and Breyer R.M. (2004) **Pharmacology and signaling of prostaglandin receptors: Multiple roles in inflammation and immune modulation**. *Pharmacol. Therapeut*. **103**, 147-166

Hawkins B.T. and Davis T.P. (2005) **The blood-brain barrier/neurovascular unit in health and disease**. *Pharmacol. Rev*. **57**, 173-185

Helliwell R.J.A., Adams L.F. and Mitchell M.D. (2004) **Prostaglandin synthases: recent developments and a novel hypothesis**. *Prostag. Leukotr. Ess.* **70**, 101-113.

Helliwell R.J.A., Berry E.B.E., O'Carroll S.J. and Mitchell M.D. (2004) **Nuclear prostaglandin receptors: role in pregnancy and parturition?** *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **70**, 149-165.

Hirase T., Staddon J.M., Saitou M., Ando-Akatsuka Y., Itoh M., Furuse M., Fujimoto K., Tsukita S. and Rubin L.L. (1997) **Occludin as a possible determinant of tight junction permeability in endothelial cells**. *J. Cell Science*. **110**, 1603-1613.

Holla V.R., Backlund M.G., Yang P., Newman R.A. and DuBois R.N. (2008) **Regulation of prostaglandin transporters in colorectal neoplasia**. *Cancer Prev. Res*. **1**, 93-99.

Hong S., Gronert K., Devchand P.R., Moussignac R-L and Serhan C.N. (2003) **Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood and glial cells**. *J. Biol. Chem*. **278**, 14677-14687.

Huber J.D., Egleton R.D. and Davis T.P. (2001) **Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier**. *Trends Neurosci*. **24**, 719-725

Izumi T., Yokomizo T., Obinata H., Ogasawara H. and Shimuzu T. (2002) **Leukotriene receptors: Classification, gene expression and signal transduction**. *J. Biochem*. **132**, 1-6.

Janzer R.C. and Raff M.C. (1987) **Astrocytes induce blood-brain properties in endothelial cells**. *Nature*. **325**, 253-257

Jaworowicz D.J., Korytko P.J., Lakhman S.S. and Boje K.M.K. (1998) **Nitric oxide and prostaglandin E2 formation parallels blood-brain barrier disruption in an experimental rat model of bacterial meningitis**. *Brain Res. Bull*. **46**, 541-546.

Jiang J., Quan Y., Ganesh T., Pouliot W.A., Dudek F.E. and Dingledine R. (2013) **Inhibition of the prostaglandin receptor EP2 following status epilepticus reduced delayed mortality and brain inflammation**. *Proc. Natl. Acad. Sci. USA*. **110**, 3591-3596.

Jones R.L., Giembycz M.A. and Woodward D.F. (2009) **Prostanoid receptor antagonists: development strategies and therapeutic applications**. *British J. Pharmacol.* **158**, 104-145.

Katsuki H. and Okuda S. (1995) **Arachidonic acid as a neurotoxic and neurotrophic substance**. *Prog. Neurobiol*. **46**, 607-636.

Kim H.-Y. (2007) **Novel metabolism of docosahexaenoic acid in neural cells**. *J. Biol. Chem*. **282**, 18661-18665.

Kniesel U. and Wolburg H. (2000) **Tight junctions of the blood-brain barrier**. *Cell. Mol. Neurobiol*. **20**, 57-76.

Kokoglu E., Tuter Y., Sandikci K.S., Yazici Z., Ulakoglu E.Z., Sonmez H. and Ozyurt E. (1998) **Prostaglandin $E_2$ levels in human brain tumor tissues and arachidonic acid levels in the plasma membrane of human brain tumors**. *Cancer Lett*. **132**, 17-21

Krause G., Winkler L., Mueller S.L., Haseloff R.F., Piontek J. and Blasig I.E. (2008) **Structure and function of claudins**. *Biochim. Biophys. Acta.* **1778**, 631-645.

Kroetz D.L. and Zeldin D.C. (2002) **Cytochrome P450 pathways of arachidonic acid metabolism**. *Curr. Opin. Lipidol*. **13**, 273-283.

Kudo I. and Murakami M. (2005) **Prostaglandin E synthase, a terminal enzyme for prostaglandin $E_2$ biosynthesis**. *J. Biochem. Mol. Biol*. **38**, 633-638.

Lai C.-H. and Kuo K.-H. (2005) **The critical component to establish in vitro BBB model: Pericyte**. *Brain Res. Rev*. **50**, 258-265.

Lai C.-H., Kuo K.-H. and Leo J.M. (2005) **Critical role of actin in modulating BBB permeability**. *Brain Res. Rev.* **50**, 7-13.

Li D.Y., Varma D.R. and Chemtob S. (1994) **Ontogenic increase in $PGE_2$ and $PGF_{2\alpha}$ receptor density in brain microvessels of pigs**. *Br. J. Pharmacol.* **112**, 59-64.

Li X., Hong S., Li P-L and Zhang Y. (2011) **Docosahexaenoic acid-induced coronary arterial dilation: Actions of 17S-hydroxy docosahexaenoic acid on $K^+$ channel activity**. *J. Pharmacol. Experimental Therapeutics*. **336**, 891-899.

Liebner S., Fischmann A., Rascher G., Duffner F., Grote E.H., Kalbacher H. and Wolburg H. (2000) **Claudin-1 and claudin-5 expression and**

**tight junction morphology are altered in blood vessels of human glioblastoma multiforme**. *Acta. Neuropathol*. **100**, 323-331.

Liebner S., Kniesel U., Kalbacher H. and Wolburg H. (2000) **Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells**. *European J. Cell Biol.* **79**, 707-717.

Mandell K.J. and Parkos C.A. (2005) **The JAM family of proteins**. *Advanced Drug Delivery Rev.* **57**, 857-867.

Mark K.S., Trickler W.J., Miller D.W. (2001) **Tumor necrosis factor-alpha induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells**. *J. Pharmacol. Exp.Ther*. **297**, 1051-1058.

Matter K. and Balda M.S. (2003) **Signalling to and from tight junctions**. *Nature Rev. Mol. Cell Biol*. **4**, 225.

McCoy J.M., Wicks J.R. and Audoly L.P. (2002) **The role of prostaglandin $E_2$ receptors in the pathogenesis of rheumatoid arthritis**. *J. Clin. Invest*. **110**, 651-658.

McGiff J.C. (1991) **Cytochrome P-450 metabolism of arachidonic acid**. *Annu. Rev. Pharmacol. Toxicol*. **31**, 339-69.

Miller D.W., Audus K.L. and Borchardt R.T. (1992) **Application of cultured endothelial cells of the brain microvasculature in the study of the blood-brain barrier**. *J. Tiss. Cult. Meth.* **14**, 217-224.

Mitchell R.W., Edmundson C.L., Miller D.W. and Hatch G.M. (2009) **On the mechanism of oleate transport across human microvessel endothelial cells**. *J. Neurochem*. **110**, 1049-1057

Mitchell R.W., On N.H., Del Bigio M.R., Miller D.W. and Hatch G.M. (2011) **Fatty acid transport protein expression in human brain and potential role in fatty acid transport across human brain microvessel endothelial cells**. *J. Neurochem*. **117**, 735-746.

Moore S.A., Yoder E., Murphy S., Dutton G.R. and Spector A.A. (1991) **Astrocytes, not neurons, produce docosahexaenoic acid (22:6ω-3) and arachidonic acid (20:4ω-6)**. *J. Neurochem.* **56**, 518-524.

Morita K., Sasaki H., Furuse M. and Tsukita S. (1999) **Endothelial claudin: Claudin-5/TMVCF constitutes tight junction strands in endothelial cells**. *J. Cell Biol.* **147**, 185-194.

Needleman P., Turk J., Jakschik B.A., Morrison A.R. and Lefkowith J.B. (1986) **Arachidonic acid metabolism**. *Ann. Rev. Biochem*. **55**, 69-102.

Negishi M., Sugimoto Y. and Ichikawa A. (1995) **Prostaglandin E receptors**. *J. Lipid Mediat. Cell.* **12**, 379-391.

O'Meara S.J., Rodgers K. and Godson C. (2007) **Lipoxins: update and impact of endogenous pro-resolution lipid mediators**. *Rev. Physiol. Biochem. Pharmacol.* **160**, 47-69.

Othman A., Ahmad S., Megyerdi S., Mussell R., Choksi K., Maddipati K.R., Elmarakby A., Rizk N. and Al-Shabrawey M. (2013) **12/15-Lipoxygenase –derived lipid metabolites induce retinal endothelial cell barrier dysfunction: Contribution of NADPH oxidase**. *PLOS One*. **8**: e57254.

Ozaki-Okayama Y., Matsumura K., Ibuki T., Ueda M., Yamazaki Y., Tanaka Y. and Kobayashi S. (2004) **Burn injury enhances brain prostaglandin $E_2$ production through induction of cyclooxygenase-2 and microsomal prostaglandin E synthase in cerebral vascular endothelial cells in rats**. *Crit. Care Med*. **32**, 795-800

Park J.Y., Pillinger M.H. and Abramson S.B. (2006) **Prostaglandin $E_2$ synthesis and secretion: The role of PGE2 synthases**. *Clin Immunol*. **119**, 229-240

Pestel, S., Jungermann, K. and Scheiferdecker, H.L. (2005) **Re-evaluation of thin layer chromatography as an alternative method for the quantification of prostaglandins from rat Kupffer cells**. *Prostagland. Other Lipid Mediat*. **75**, 123-139.

Peters-Golden M. and Henderson W.R. (2007) **Leukotrienes**. *N. Engl. J. Med*. **357**, 1841-1854.

Picq M., Chen P., Perez M., Michaud M., Vericel E., Guichardant M. and Lagarde M. (2010) **DHA metabolism: Targeting the brain and lipoxygenation**. *Mol. Neurobiol*. **42**, 48-51.

Rao R. (2009) **Occludin phosphorylation in regulation of epithelial tight junctions**. *Ann. N. Y. Acad. Sci*. **1165**, 62-68.

Rapoport S.I. (2008) **Arachidonic acid and the brain**. *J. Nutr*. **138**, 2515-2520

Rapoport S.I., Chang M.C.J. and Spector A.A. (2001) **Delivery and turnover of plasma-derived essential PUFAs in mammalian brain**. *J. Lipid Res.* **42**, 678-685.

Reid G., Wielinga P, Zelcer N, van der Heijden I, Kuil A, de Haas M, Wijnholds J and Borst P. (2003) **The human multidrug resistance protein MRP4 functions as a prostaglandin efflux transporter and is inhibited by nonsteroidal anti-inflammatory drugs**. *PNAS*. **100**, 9244-9249.

Roman R.J. (2002) **P-450 metabolites of arachidonic acid in the control of cardiovascular function**. *Physiol. Rev*. **82**, 131-185.

Rubin L.L., Hall D.E., Porter S., Barbu K., Cannon C., Horner H.C., Janatpour M., Liaw C.W., Manning K., Morales J., Tanner L.I., Tomaselli K.J. and Bard F. (1991) **A cell culture model of the blood-brain barrier**. *J. Cell Bio*. **115**, 1725-1735.

Russel F.G.M., Koenderink J.B. and Masereeuw R. (2008) **Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signaling molecules**. *Trend Pharmacol. Sci*. **29**, 200-207.

Saini-Chohan H.K., Dakshinamurti S., Taylor W.A., Shen G.X., Murphy R., Sparagna G.C. and Hatch G.M. (2011) **Persistent pulmonary hypertension results in reduced tetralinoleoyl-cardiolipin and mitochondrial complex II + III during the development of right ventricular hypertrophy in the neonatal pig heart**. *Am. J. Physiol. Heart Circ. Physiol.* **301**, H1415-24.

Samuelsson B, Dahlen S-E, Lindgren J, Rouzer C.A. and Serhan C.N. (1987) **Leukotrienes and lipoxins: Structures, biosynthesis and biological effects**. *Science*. **237**, 1171-1176.

Samuelsson B., Morgenstern R. and Jakobsson P.-J. (2007) **Membrane prostaglandin E synthase-1: A novel therapeutic target**. *Pharmacol. Rev*. **59**, 207-224.

Schuster V.L. (1998) **Molecular mechanisms of prostaglandin transport**. *Annu. Rev. Physiol*. **60**, 221-242.

Schwab J.M. and Serhan C.N. (2006) **Lipoxins and new lipid mediators in the resolution of inflammation**. *Curr. Opin. Pharmacol*. **6**, 414-420.

Serhan C.N. (2005) **Novel eicosanoids and docosanoid mediators: resolvins, docosatrienes and neuroprotectins**. *Curr. Opin. Clin. Nutr. Metab. Care*. **8**, 115-121.

Serhan C.N. (2005) **Novel ω-3-derived local mediators in anti-inflammation and resolution**. *Pharmacol. Therapeutics*. **105**, 7-21.

Serhan C.N. (2007) **Resolution phase of inflammation: Novel endogenous anti-inflammatory and proresolving lipid mediators and pathways**. *Annu. Rev. Immunol*. **25**, 101-37.

Serhan C.N. and Chiang N. (2008) **Endogenous pro-resolving and anti-inflammatory lipid mediators: a new pharmacologic genus**. *British J. Pharmacol*. **153**, S200-S215.

Serhan C.N., Gotlinger K., Hong S. and Arita M. (2004) **Resolvins, docosatrienes and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis**. *Prostaglandins and other lipid mediators.* **73**, 155-172.

Shimizu T. and Wolfe L.S. (1990) **Arachidonic acid cascade and signal transduction**. *J. Neurochem*. **55**, 1-15

Smith W.L. (1989) **The eicosanoids and their biochemical mechanisms of action**. *Biochem. J*. **259**, 315-324

Smith W.L. and Murphy R.C. (2008) **The eicosanoids: cyclooxygenase, lipoxygenase and epoxygenase pathways**. In *Biochemistry of Lipids, Lipoproteins and membranes*, ed. Vance D.E. and Vance J.E., pp.331-362. Elsevier, Amsterdam, The Netherlands.

Sonoda N., Furuse M., Sasaki H., Yonemura S., Katahira J., Horiguchi Y. and Tsukita S. (1999) ***Clostridium perfringens*** **enterotoxin fragment removes specific claudins from tight junction strands: Evidence for direct involvement of claudins in tight junction barrier**. *J. Cell Biol*. **147**, 195-204.

Spector A.A. (2009) **Arachidonic acid cytochrome P450 epoxygenase pathway**. *J. Lipid Res*. **50**, S52-S56

Stanimirovic D. and Satoh K. (2000) **Inflammatory mediators of cerebral endothelium: A role in ischemic brain inflammation**. *Brain Pathol*. **10**, 113-126.

Stuhlmeier K.M, Tarn C., Csizmadia V. and Bach F.H. (1996) **Selective suppression of endothelial cell activation by arachidonic acid**. *Eur. J. Immunol*. **26**, 1417-1423.

Trickler W.J., Mayhan W.G. and Miller D.W. (2005) **Brain microvessel endothelial cell responses to tumor necrosis factor-alpha involve a nuclear factor kappa B (NF-κB) signal transduction pathway**. *Brain Research*. **1048**, 24-31

Turini M.E. and Dubois R.N. (2002) **Cyclooxygenase-2: A therapeutic target**. *Annu. Rev. Med*. **53**, 35-57.

Verma S., Kumar M., Gurjav U., Lum S. and Nerurkar V.R. (2010) **Reversal of West Nile virus-induced blood-brain barrier disruption and tight junction proteins degradation by matrix metalloproteinases inhibitor**. *Virology*. **397**, 130-138.

de Vries H.E., Blom-Roosemalen M.C.M., de Boer A.G., van Berkel T.J.C., Breimer D.D. and Kuiper J. (1996) **Effect of endotoxin on permeability of bovine cerebral endothelial cell layers *in vitro***. *J. Pharmacol. Exp. Ther*. **277**, 1418-1423.

Williams J.R., Leaver H.A., Ironside J.W., Miller E.P., Whittle I.R. and Gregor A. (1998) **Apoptosis in human primary brain tumours: actions of arachidonic acid**. *Prostag. Leukotr. Ess*. **58**, 193-200.

Yakubu M.A. and Leffler C.W. (2005) **Regulation of cerebral microvascular endothelial cell cyclooxygenase-2 message and activity by blood derived vasoactive agents**. *Brain Res. Bulletin*. **68**, 150-156.

Yang Y., Estrada E.Y., Thompson J.F., Liu W. and Rosenberg G.A. (2007) **Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat**. *J. Cerebral Blood Flow Metab*. **27**, 697-709.

Yerram N.R., Moore S.A. and Spector A.A. (1989) **Eicosapentaenoic acid metabolism in brain microvessel endothelium: effect on prostaglandin formation**. *J. Lipid Res*. **30**, 1747-1757.

Zeldin D.C. (2001) **Epoxygenase pathways of arachidonic acid metabolism**. *J. Biological Chem.* **276**, 36059-36062.

Zlokovic B.V. (2008) **The blood-brain barrier in health and chronic neurodegenerative disorders**. Neuron. **57**, 178-201.