Elongation factor 1A-1 and hepatocyte response to fatty acid excess

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Graduate Program in Physiology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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ELONGATION FACTOR 1A-1 AND HEPATOCYTE RESPONSE TO FATTY ACID EXCESS

(Thesis format: Monograph)

by

Alexandra Manuela Stoianov

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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ABSTRACT

Obesity is associated with elevated levels of serum fatty acids, which accumulate in non-adipose tissues including the liver. Elongation factor 1A-1 (EF1A-1) has previously been shown to participate in the cell stress and death response of cardiomyocytes to excess saturated fatty exposure, and in steatotic mouse myocardium. In this thesis, the hypothesis that the hepatocyte response to fatty acid overload involves EF1A-1 was tested. EF1A-1 expression was induced in the livers of obese mice in association with severe hepatic steatosis, and in HepG2 human hepatoma cells in response to excess palmitate. Partial translocation of EF1A-1 from the ER to polymerized actin preceded palmitate-induced cell death. Inhibiting the elongation function of the protein using a specific inhibitor decreased palmitate-induced cell death. Results indicate EF1A-1 participates in hepatocyte stress response to saturated fatty acid excess possibly by mediating changes in protein synthesis related to actin cytoskeleton remodeling that occur during cell stress.

Keywords: Elongation factor 1A-1, obesity, NAFLD, lipotoxicity, hepatic steatosis
CO-AUTHORSHIP STATEMENT

Plasma and liver lipid measurements in Table 3.1 were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute by Cindy Sawyez and Brian Sutherland.

Immunofluorescence confocal images in Figures 3.7 A-F and 3.8 A-F were taken by Debra Robson.

Subcellular fractionation and immunoblotting in Figure 3.7 H was done by Dr. Nica Borradaille.
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Tissue and plasma samples from C57BL/6J and ob/ob mice were kindly provided by Dr. Murray Huff and former members of the Huff laboratory, Dr. Erin Mulvihill and Julia Assini, MSc.
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<tr>
<td>aa-tRNA</td>
<td>Aminoacyl-tRNA</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchnonic acid</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CHOP</td>
<td>C/EBP-homologous protein</td>
</tr>
<tr>
<td>ChREBP</td>
<td>Carbohydrate response element binding protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computerized tomography</td>
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<tr>
<td>CYP2E1</td>
<td>Cytochrome P450 2E1</td>
</tr>
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<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
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<tr>
<td>DGAT2</td>
<td>Diacylglycerol acyltransferase 2</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>eEF1A-1</td>
<td>Eukaryotic elongation factor 1A-1</td>
</tr>
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<td>eEF1B</td>
<td>Eukaryotic elongation factor 1B</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Eukaryotic translation initiation factor 2α</td>
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<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagles minimum essential medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>G-actin</td>
<td>Globular actin</td>
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<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GRP78</td>
<td>Glucose-regulated protein 78</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IRE1α</td>
<td>Inositol-requiring enzyme 1α</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>JNK</td>
<td>c-jun-N-terminal kinase</td>
</tr>
<tr>
<td>LSB</td>
<td>Laemmli sample buffer</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NASH</td>
<td>Nonalcoholic steatohepatitis</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBA</td>
<td>4-Phenyl butyric acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PERK</td>
<td>Double-stranded RNA-dependent like ER kinase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCD1</td>
<td>Stearoyl-CoA desaturase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element-binding protein 1c</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>XBP-1</td>
<td>X box binding protein 1</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
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</table>
1.0 INTRODUCTION
1.1 Obesity

In recent decades, the prevalence of obesity has increased alarmingly, making it a significant health problem in not only high-income countries, but low and middle-income countries as well (World Health Organization, 2013). Obesity is defined as abnormal or excessive fat accumulation that presents a risk to one’s health. A simple measurement of obesity is the body mass index (BMI), which is calculated by dividing a person’s weight (in kilograms) by the square of his or her height (in metres) (Pasco et al., 2012). A person with a BMI greater than 25 is considered overweight, while having a BMI greater than 30 is generally considered obese (Pasco et al., 2012). The primary cause of obesity is an energy imbalance between calories consumed and calories expended. According to statistics by The World Health Organization, obesity has more than doubled since 1980 and today, over 500 million adults are obese worldwide. In Canada, 19.8% of men and 16.8% of women over the age of 18 have a BMI of 30 or greater according to data collected from 2011 (Statistics Canada). Obesity has been recognized as an important risk factor for many other conditions including insulin resistance and type 2 diabetes, hypertension, dyslipidemia, coronary disease, heart failure, and cancer. A subset of these conditions have been clustered and described as the metabolic syndrome, which is an insulin-resistant state characterized by various cardiovascular risk factors (Johnson and Weinstock, 2006). The current unified criteria for metabolic syndrome takes into account definitions from several international organizations (Alberti et al., 2009). These criteria are abdominal obesity (increased waist circumference) plus two of the following characteristics: elevated plasma triglycerides (≥ 1.7 mmol/l), elevated fasting blood glucose (≥ 5.6 mmol/l), decreased high-density lipoprotein (HDL) cholesterol (< 1.03 mmol/l), and elevated blood pressure (≥ 130/85) (Alberti et al., 2009).
The majority of cells have limited ability to store excess lipids and to meet their own fuel needs during extended famine. As a result, specialized cells (adipocytes) have evolved to store fuel during caloric surplus and distribute lipids in times of need (Unger et al., 2010). When caloric intake exceeds caloric expenditure, adipocytes initially undergo hypertrophy. This process triggers adipose tissue paracrine signaling to stimulate adipogenesis in order to maintain adipose tissue physiological functions during increased energy storage (Bays et al., 2008). In addition to storing excess calories as triglycerides, adipocytes secrete leptin, a hormone that signals satiety and reduces caloric intake. Leptin is also responsible for stimulating fatty acid oxidation in non-adipose tissues (Unger et al., 2010). Eventually, during conditions of chronic energy overload, the capacity of adipose tissue to expand and function fails leading to adipocyte dysfunction and uncontrolled lipolysis. This results in elevated circulating free fatty acids (Muoio and Newgard, 2006). In the liver, hepatocytes package and secrete excess lipids as very low-density lipoprotein (VLDL), however, chronic exposure of the liver to circulating free fatty acids (FFA) leads to hepatic insulin resistance. This impairs hepatic ability to downregulate VLDL production in response to insulin, resulting in VLDL overproduction and hypertriglyceridemia (Sparks et al., 2012). As a result of this increased flux of lipid from adipose and liver to peripheral tissues, fatty acids begin to accumulate in non-adipose locations such as the heart and cardiovascular system, skeletal muscle, kidney, pancreas (Brookheart et al., 2009) (Figure 1.1). Ectopic lipid accumulation can lead to insulin resistance in those tissues, inflammation, and apoptosis. The process leading from lipid accumulation to cell dysfunction and death is termed lipotoxicity, which occurs when cells can no longer oxidize, safely store or export excess lipid (Unger, 2003). Although dietary lipids are important for normal biological functions such as membrane biosynthesis, protein
Figure 1.1 Obesity-related perturbations in metabolic control lead to ectopic lipid accumulation in multiple organs. A high calorie, high fat diet coupled with inactivity leads to elevated circulating free fatty acids (FA) and triglycerides (TG). During conditions of chronic energy overload, the capacity of adipose tissue to expand and function fails. As a result, lipids accumulate in non-adipose tissues such as the heart and cardiovascular system, skeletal muscle, kidney, pancreas and liver (indicated by yellow circles). In the liver, chronic exposure to excess free fatty acids impairs its ability to downregulate VLDL production, resulting in VLDL overproduction and hypertriglyceridemia. (Adapted from Muoio and Newgard, 2006)
modification, intracellular signaling and energy homeostasis, chronic exposure of organs to elevated concentrations of these lipids leads to organ dysfunction and disease development (Szendroedi and Roden, 2009).

### 1.2 NAFLD

Dietary intake of a high calorie and high fat diet coupled with low physical activity leads to increased circulating levels of free fatty acids and storage of triglycerides in ectopic organs, including the liver (Szendroedi and Roden, 2009). In the absence of alcohol, lipid accumulation within the liver is termed nonalcoholic fatty liver disease (NAFLD) and is considered the hepatic manifestation of the metabolic syndrome. NAFLD describes the chronic liver disease associated with hepatocyte lipotoxicity and is the most common form of liver disease affecting both adults and children throughout the world (Erickson, 2009). The prevalence of NAFLD is increasing due to the increasing rates of obesity, type 2 diabetes, and metabolic syndrome, all of which are risk factors for the development of NAFLD. It represents a range of hepatic disease from simple steatosis, which is the accumulation of fat in the liver and is considered mostly benign, to nonalcoholic steatohepatitis (NASH), characterized by inflammation and hepatocyte injury (Utzschneirder and Kahn, 2006). NASH can progress to fibrosis and cirrhosis, and ultimately end stage liver disease. As a result, NAFLD is becoming an increasingly common cause of liver transplantation as well as a risk factor for the progression of hepatocellular carcinoma, primarily due to cirrhosis associated with NAFLD (Shimada et al., 2002; Bullock et al., 2004).

Most patients with fatty liver are asymptomatic and when symptoms do occur, they are usually non-specific. NAFLD diagnosis remains complex and is usually discovered when a
condition other than fatty liver is suspected (Schreuder et al., 2008). The main histological characteristics of NASH, in addition to steatosis, include hepatocyte ballooning, lobular inflammation and displacement of nuclei to the edge of the cell. The presence of fibrosis indicates progression of the disease to more severe liver injury (Yeh and Brunt, 2007). Existing non-invasive diagnostic markers of NAFLD include a set of clinical signs and symptoms, imaging tests and blood test results (Wieckowska and Feldstein, 2008). Elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are primarily observed in patients with NAFLD. These enzymes are located within hepatocytes and are released into circulation upon liver injury. ALT is located predominantly in the liver, while AST is also found in other tissues and can be elevated in diseases affecting organs such as the heart or skeletal muscle (Ozer et al., 2008). However, the reliability of these tests has been debated due to the fact that levels fluctuate within the normal limit in the majority of patients with NASH (Adams and Feldstein, 2011). Common imaging techniques to diagnose hepatic steatosis include ultrasound, computerized tomography (CT) scan and magnetic resonance imaging (MRI). The most commonly used and least expensive method is ultrasound, while MRI is the most accurate available imaging technique for quantification of hepatic steatosis (Schreuder et al., 2008). Nonetheless, no imagining technique is currently capable of detecting the subtle histological changes, ballooning and inflammation that distinguish hepatic steatosis from early NASH (Adams and Feldstein, 2011). Liver biopsy remains the only reliable method to accurately diagnose NASH and to determine the severity of fibrosis and liver injury.

The cause and progression of NAFLD is complex and not fully understood. However, insulin resistance is a key feature of ectopic lipid accumulation in the liver. NAFLD is linked
to both hepatic and adipose tissue insulin resistance in addition to a reduction in whole-body insulin sensitivity (Utzschneider and Kahn, 2006). Under normal physiological conditions, insulin suppresses lipolysis in adipose tissue by inhibiting hormone-sensitive lipase (HSL). In insulin-resistant states, lipolysis is increased and esterification of fatty acids is inhibited, resulting in excess flux of free fatty acids to the liver (Trauner et al., 2010). The associated hyperinsulinemia enhances hepatic de novo lipogenesis through activation of transcription factors such as sterol regulatory element-binding protein 1c (SREBP-1c), which leads to the transcriptional activation of lipogenic genes. Furthermore, hyperglycemia induces carbohydrate response element binding protein (ChREBP) expression, which leads to the transcriptional activation of additional lipogenic genes (Trauner et al., 2010). The actions of these two transcription factors lead to activation of enzymes required for the conversion of excess glucose to fatty acids, which can be esterified into triglycerides (Trauner et al., 2010).

Hepatic steatosis does not always progress to liver injury, indicating that other factors, including environmental and genetic determinants, are necessary. It is not known why some individuals with only hepatic steatosis progress to NASH while others do not. Some have suggested that the accumulation of excess triglyceride (hepatic steatosis) and lipotoxic cell damage are two responses that occur simultaneously, but independently (Neuschwander-Tetri, 2010).

1.3 Lipotoxicity in Hepatocytes

In the early stages of NAFLD, triglycerides accumulate in hepatocytes. Serum free fatty acids are elevated in patients with NASH and are correlated with disease progression (Nehra et al., 2001). There are three main sources of hepatic lipids. Free fatty acids released
from adipose tissue contribute to the majority of hepatic triglyceride content and play a critical role in the development of NAFLD (Donnelly et al., 2005). *De novo* lipogenesis is the second most important mechanism, while dietary lipid provides about 10% of the hepatic lipid content (Ibrahim et al., 2011).

Free fatty acids are carried in the circulation bound to albumin, which maintains a low unbound concentration and allows a higher concentration of free fatty acids to be carried in the serum (Bradbury, 2006). They can enter the cell by simple diffusion, which is most likely when circulating, or extracellular, concentrations are very high, or by facilitated transport. Several fatty acid transport proteins (FATP) and the fatty acid uptake transporter CD36 have been identified to facilitate uptake (Pohl et al., 2004; Zhou et al, 2008). FATP5 is exclusively expressed in hepatocytes and is involved with uptake of long-chain (greater than 16 carbons) free fatty acids (Doege et al., 2006). Once in the cell, free fatty acids may be converted to triglycerides or undergo β-oxidation at the mitochondria. Triglycerides may be targeted for export through the production of VLDL particles, or for storage in lipid droplets (Bradbury, 2006). In hepatocytes overwhelmed by excess lipids, in particular saturated fatty acids, multiple lipotoxic responses are activated resulting in oxidative (Li et al., 2008; Wu et al., 2008) and endoplasmic reticulum (ER) stress (Wei et al., 2006; Lee at al., 2010), and ultimately cell death.

### 1.3.1 Saturated and Unsaturated Fatty Acids

In recent studies, *in vitro* models of steatosis have been used to study the consequences of excess lipid accumulation in hepatocytes. These studies typically involve hepatocyte cell lines or primary hepatocytes treated with high pathophysiological concentrations of saturated
and unsaturated fatty acids conjugated to bovine serum albumin (BSA). Numerous studies have demonstrated that saturated fatty acids are more harmful to hepatocytes than unsaturated fatty acids (Wei et al., 2006; Malhi et al., 2006; Li et al., 2009). This may be due to their relative inefficiency, compared to unsaturated fatty acids, to be esterified and stored safely as triglycerides (Listenberger et al., 2003). Treating cells simultaneously with both saturated and unsaturated fatty acids rescues cells from saturated fatty acid induced apoptosis by diverting potentially toxic saturated fatty acids to triglyceride formation (de Vries et al., 2007; Akazawa et al., 2010). Similar observations have been made in animal models of NAFLD suggesting that saturated fatty acids are damaging to the liver while unsaturated fatty acids are not (Wang et al., 2006; Li et al., 2009). In leptin-receptor deficient db/db mice fed a methionine and choline deficient diet, inhibition of diacylglycerol acyltransferase 2 (DGAT2), the enzyme responsible for esterification of free fatty acids into triglycerides, decreased hepatic steatosis but elevated hepatic free fatty acids and markers of oxidative stress, inflammation and fibrosis (Yamaguchi et al., 2007). Mice with hepatic DGAT2 overexpression develop hepatic steatosis with increased amounts of triglycerides in the liver, but no evidence of liver damage or insulin resistance (Monetti et al., 2007). Genetic or pharmacological inhibition of stearoyl-CoA desaturase-1 (SCD1), the enzyme that converts saturated fatty acids to monounsaturated fatty acids, renders hepatocytes more sensitive to saturated fatty acid-induced apoptosis (Li et al., 2009). SCD1−/− mice fed a methionine and choline deficient diet accumulated less triglycerides in the liver compared to wild-type mice but have increased serum free fatty acids, hepatocyte apoptosis, liver damage and fibrosis. Meanwhile, mice fed a methionine and choline deficient diet supplemented with oleate, a monounsaturated fatty acid, have less severe liver injury and apoptosis (Li et al., 2009). Taken together, this suggests that saturated fatty acids are primarily
responsible for hepatocyte lipotoxicity, while their esterification into triglycerides serves as a protective mechanism.

1.3.2 ER Stress

The ER is responsible for many cellular functions such as protein synthesis, lipid synthesis, carbohydrate metabolism, calcium homeostasis and drug detoxification. It also plays an important role in sensing cellular stress (Fu et al., 2012). Physiological states that disrupt ER homeostasis create an imbalance between the protein-folding load and the ER’s capacity to fold proteins, resulting in unfolded or misfolded proteins that accumulate in the ER lumen (ER stress). To ensure the accuracy of protein folding and to prevent accumulation of such proteins, eukaryotic cells have developed the unfolded protein response (UPR). The UPR is a cellular mechanism of inhibiting protein synthesis, increasing protein folding and increasing ER-associated protein degradation in order to reduce the ER load of unfolded protein (Kaplowitz et al., 2007). This response is mediated by 3 ER-localized protein sensors: inositol-requiring enzyme 1α (IRE1α), double-stranded RNA-dependent like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Fu et al., 2012). Each protein sensor spans the ER membrane and consists of 3 domains; an ER-luminal domain that senses unfolded proteins, a transmembrane domain and a cytosolic domain that conveys signals to the transcriptional or translational apparatus. Under normal physiological conditions, these ER sensors are associated with the protein folding chaperone glucose-regulated protein 78 (GRP78) (also known as binding immunoglobulin protein, BiP) at their luminal domains and maintained in an inactive state (Fu et al., 2012). Under conditions of ER stress, GRP78 binds to unfolded or misfolded proteins, resulting in the release and subsequent activation of the ER
stress sensors. Following the release of GRP78 from PERK, PERK phosphorylates eukaryotic translation initiation factor 2 α (eIF2α). Phosphorylation of eIF2α blocks global protein synthesis while selectively increasing translation of certain mRNAs, such as activating transcription factor 4 (ATF4). ATF4 promotes apoptosis under severe stress conditions (Lu et al., 2012). In response to ER stress, IRE1α, by autophosphorylation, initiates expression of the spliced form of X box binding protein 1 (XBP-1) which activates the transcription of genes encoding protein folding chaperones and factors that regulate maturation, secretion, and ER-associated protein degradation. Meanwhile, the release of GRP78 from ATF6 allows ATF6 to translocate to the Golgi apparatus, become cleaved and, similar to XBP-I, activate the expression of UPR target genes (Fu et al., 2012). If ER stress is prolonged or the UPR is unable to maintain homeostasis, the ER stress response is initiated, which induces proinflammatory and proapoptotic pathways. These include activation of c-jun-N-terminal kinase (JNK) and increased C/EBP-homologous protein (CHOP) expression, which go on to activate apoptotic pathways (Kaplowitz et al., 2007). ER-stress induced apoptosis can involve mitochondria, which are recruited by ER signals such as calcium release. Increased expression of GRP78 and CHOP are characteristic markers of the UPR and the ER stress response, respectively, and have been shown to be activated under conditions of ER stress in hepatocytes both \textit{in vitro} and \textit{in vivo} (Kaplowitz et al., 2007). In the present study, GRP78 is used as a marker of the UPR in hepatocytes.

Increasing evidence suggests that ER stress acts as an upstream signal in saturated fatty acid-induced apoptosis in many cell types, including hepatocytes. Chronic exposure of cultured hepatocytes to long-chain saturated fatty acids leads to upregulation of UPR and ER stress markers (CHOP, GRP78, phosphorylated PERK and phosphorylated eIF2α), followed
by cell death (Wei et al., 2006; Wei et al., 2009; Lee et al., 2010). Mechanisms of saturated fatty acid-induced ER stress involve release of ER luminal calcium stores and activation of caspases (Wei et al., 2009; Wei et al., 2007). In vivo, both dietary and genetic models of hepatic steatosis are associated with upregulation of GRP78, CHOP, phosphorylated PERK and XBP-1 splicing (Ozcan et al., 2004; Wang et al., 2006; Rahman et al., 2007). Furthermore, saturated fatty acids can cause direct induction of ER stress by becoming incorporated into the saturated phospholipid and triglyceride species of the ER membrane, thereby compromising its structure and function (Borradaile et al., 2006b).

1.3.3 Oxidative Stress

In addition to ER stress, recent studies have suggested that oxidative stress, indicated by elevated levels of reactive oxygen species (ROS), is involved in the hepatocyte lipotoxic response (Li et al., 2008; Wu et al., 2008; Li et al. 2009). ROS are involved in cellular signaling and homeostasis. However, cell stress, mitochondrial dysfunction and decreased antioxidant defenses can lead to the overproduction of ROS (Leamy et al., 2013). This can cause damage to proteins, lipids and DNA, as well as compromise membrane integrity and organelle function, thereby inducing cell death (Brookheart et al., 2009). The elevated levels of ROS associated saturated fatty overload in hepatocytes (Li et al., 2008) can be attributed to various sources. Possible mechanisms include increased expression of cytochrome P450 2E1 (CYP2E1) (the enzyme responsible for the oxidative metabolism of various substrates), upregulation of NADPH oxidase and increased mitochondrial β-oxidation (Aubert et al., 2011; Lambertucci et al., 2008; Inoguchi et al., 2000; Cacicedo et al., 2005; Nakamura et al., 2009). However, the exact role of β-oxidation in promoting lipotoxic ROS accumulation is
unclear, suggesting that lipids play a complex role in generating ROS, which likely involves multiple mechanisms (Leamy et al., 2013).

1.3.4 Mitochondrial Dysfunction

Studies using primary mouse hepatocytes and HepG2 cells show that supplementation with long chain free fatty acids results in Bcl-2-associated X protein (Bax) translocation to the lysosome, lysosomal permeabilization and activation of cathepsin B, a lysosomal cysteine protease. This is followed by mitochondrial dysfunction, characterized by mitochondrial depolarization, cytochrome c release into the cytosol and increased generation of ROS (Li et al., 2008). Pharmacological inhibition of cathepsin B activation and mitochondrial cytochrome c release results in decreased free fatty acid induced lipid accumulation and hepatocyte apoptosis (Wu et al., 2008; Cheng et al., 2009). Moreover, in liver tissue of patients with NASH, mitochondrial function is impaired, as reflected by impaired mitochondrial respiratory chain activity (Pérez-Carreras et al., 2003). In addition to these functional abnormalities, structural changes, such as longitudinal or spherical swelling and the development of intramitochondrial crystals, are observed in patients with steatohepatitis. It is thought that intramitochondrial crystals consist of phospholipids, and are perhaps formed in response to oxidative stress (Caldwell et al., 2009). Taken together, these findings suggest that mitochondrial dysfunction plays a role in the pathogenesis of lipotoxicity.

ER stress is closely linked to oxidative stress (Zhang and Kaufman, 2008). Oxidative stress-induced apoptosis requires the release of calcium stores from the ER lumen, which can lead to ER stress by impairing normal protein-folding functions (Scorrano et al., 2003; Rao et al., 2004). Together they lead to apoptosis by mitochondrial pathways and caspase activation
(Wei et al., 2006; Li et al., 2008). A general model for lipotoxic cell death incorporating the pathways described above is shown in Figure 1.2.
Figure 1.2 Pathways involved in the cellular response to saturated fatty acid overload.

During exposure to excess saturated fatty acids, cellular capacities to store them as triglycerides (lipid droplets) and to catabolize them through β-oxidation (mitochondria) are overwhelmed. The resultant production of ROS, from several potential sources, can induce ER stress. Palmitate can also be rapidly incorporated into complex lipids in the ER membrane resulting in dramatic impairment of the structure and integrity of the ER. Both oxidative stress and altered ER composition and integrity result in the release of ER calcium stores, triggering apoptotic cell death via mitochondria.
1.4 Eukaryotic elongation factor 1A-1

Eukaryotic elongation factor 1A-1 (eEF1A-1) was originally discovered as the factor responsible for catalyzing the binding of aminoacyl-tRNA (aa-tRNA) to the A-site of the ribosome during the elongation phase of protein synthesis (McKeehan and Hardesty, 1969), but is now known to mediate other cellular processes including protein degradation, apoptosis and cytoskeletal organization (Figure 1.3) (Mateyak and Kinzy, 2010). eEF1A-1 is one of two isoforms, the other being eEF1A-2, that share 92% amino acid sequence similarity (Kahns et al., 1998). Both variants have similar functions with regards to peptide elongation, however they are differentially expressed (Kahns et al., 1998). eEF1A-1 is expressed ubiquitously in mouse and human tissues (Kahns et al., 1998; Knudsen et al., 1993) while eEF1A-2 is expressed tissue-specifically in skeletal muscle, brain and heart (Lee et al., 1992; Khalyfa et al., 2001). The liver exclusively expresses only the eEF1A-1 isoform (Knudsen et al., 1993; Newbery et al., 2007).

1.4.1 Canonical role of eEF1A-1

eEF1A-1 is activated upon GTP binding through a conformational change that triggers recruitment of aa-tRNA. This complex then binds to the A-site of the ribosome, resulting in a quaternary complex. GTP is hydrolyzed to GDP following correct codon-anticodon interaction which requires the activity of eEF1B, a guanine nucleotide exchange factor (GEF), to promote GDP release and reactivation of the protein through passive binding of GTP (Mateyak and Kinzy, 2010). The phosphorylation status eEF1A-1 has been shown to serve as a regulatory mechanism for controlling protein synthesis at the elongation phase. For example, phosphorylation of eEF1A-1 at Ser300 by TGF-β inhibits protein synthesis by disrupting
Figure 1.3 Canonical and non-canonical functions of eEF1A-1. The canonical function of eEF1A-1 is to bind aa-tRNA in a GTP-dependent manner and deliver it to the ribosome. The non-canonical functions include turnover of misfolded proteins, binding and bundling the actin cytoskeleton as well as other cytoskeletal components, and apoptosis. (Adapted from Mateyak and Kinzy, 2010)
binding to aa-tRNA (Lin et al., 2010). eEF1A-1 consists of three domains (I, II and III): domain I is the GTP-binding domain, domain II associates with the aminoacyl of aa-tRNA and domain III is primarily involved with binding to actin (Mateyak and Kinzy 2010). eEF1A-1 is abundant within the cell, comprising 1-2% of total cellular protein in normal growing cells (Ejiri et al., 2002). However, cellular levels of eEF1A-1 are not rate limiting for protein synthesis (Condeelis, 1995). Its high cellular concentration and proximity to the ribosome suggests a role for eEF1A-1 in the control and accuracy of peptide elongation. This is supported by data that eEF1A-1 plays a role in the quality control of newly-synthesized proteins by binding nascent or unfolded proteins that are no longer associated with the ribosome, but not to correctly folded proteins (Hotokezaka et al., 2002). eEF1A-1 was identified as a factor required for the degradation of N alpha-acetylated proteins (Gonen et al., 2004) and has been shown to bind damaged nascent proteins that are ubiquinated to facilitate their delivery to the proteasome (Chuang et al., 2005). Studies investigating the intracellular localization of eEF1A-1 indicate it is predominantly localized in the cytosolic compartment associated with endoplasmic reticulum, and specifically ribosomes (Hayashi et al., 1989; Minella et al., 1996; Didichenko et al., 1991).

1.4.2 Association with the Cytoskeleton

In addition to its function in peptide elongation, recent studies have presented evidence for a role of eEF1A-1 in cytoskeletal reorganization. eEF1A-1 was originally identified as an actin-binding protein by Yang et al (1990). In this study, it was shown that eEF1A-1 can bind the actin cytoskeleton in a reversible manner (Yang et al., 1990). Others have indicated that eEF1A-1 localizes with F-actin by fluorescence microscopy (Dharmawardhane et al., 1991;
Collings et al., 1994). eEF1A-1 regulates the actin cytoskeleton through its G-actin binding and F-actin bundling activity, and has been demonstrated to alter the rate of actin polymerization (Gross and Kinzy, 2005; Murray et al., 1996; Doyle et al., 2011). Furthermore, studies using a genetic screen have identified the specific regions of eEF1A-1 responsible for actin binding, which are primarily located in domain III. Mutations in these regions lead to reduced actin binding and disorganization of the actin cytoskeleton (Gross and Kinzy, 2005, 2007; Liu et al., 2002). It has also been suggested that eEF1A-1 functions in microtubule dynamics. In vitro experiments demonstrate that eEF1A-1 can bundle and stabilize microtubules (Durso and Cyr, 1994; Moore et al., 1999), while others have indicated it is involved with the severing of microtubules (Shiina et al., 1994).

1.4.3 Role in Apoptosis

eEF1A-1 has been reported to play an important role in mediating apoptosis. Early studies in mouse fibroblasts show that eEF1A-1 protein levels correlate with the rate of apoptosis during conditions of cell stress. In fact, eEF1A-1 overexpression accelerates the rate of apoptosis (Duttaroy et al., 1998). Both oxidative and ER stress induce its expression and it plays an important role in mediating apoptosis under these conditions (Chen at al., 2000; Borradaile et al., 2006a). Furthermore, recent evidence suggests a role for eEF1A-1 in anoikis (apoptosis due to loss of cell anchorage) (Itagaki et al., 2012). eEF1A-1 overexpression at the plasma membrane increases susceptibility of fibroblasts to anoikis during serum starvation conditions, while disrupting the increase in eEF1A-1 at the plasma membrane by small interfering RNA (siRNA) renders cells more resistant to anoikis (Itagaki et al., 2012). The pro-apoptotic activity of eEF1A-1 appears to depend on its ability to regulate the cytoskeleton
(Shiina et al., 1994; Kato et al., 1997; Borradaile et al., 2006a), which undergoes dramatic changes during apoptosis (Ndozangue-Touriguine et al., 2008).

1.5 Objectives and Hypothesis

Through a genetic screen using a ROSAβgeo retroviral promoter trap in CHO cells to identify factors that, when disrupted, confer resistance to saturated fatty acid-induced apoptosis, eEF1A-1 was identified as a mediator of lipotoxic cell death (Borradaile et al., 2006a). In isolated cardiomyocytes exposed to excess palmitate, eEF1A-1 protein expression increased in association with oxidative stress, and ER stress. Oxidative stress, ER stress, and induction of eEF1A-1 all preceded apoptotic cell death. Knocking down eEF1A-1 expression conferred resistance to palmitate-induced cell death through the prevention of changes in polymerized actin content that normally precede apoptosis (Borradaile et al., 2006a). *In vivo*, eEF1A-1 expression is induced in steatotic myocardium of mice with cardiac-specific lipotoxicity, in association with upregulation of markers of oxidative and ER stress (Borradaile et al., 2006a).

Both the heart and the liver are key organs in the metabolism of fatty acids, and are significantly impacted by the progressive steatosis that occurs during obesity and metabolic disease (Szendroedi and Roden, 2009). The studies in this thesis extend the previous findings in cardiomyocytes, outlined above, to hepatocytes and steatotic liver.

**Hypothesis:** eEF1A-1 protein responds to lipid overload induced stress in hepatocytes and in the livers of obese mice, and participates in the process of lipotoxicity. Furthermore, the mechanism of action of eEF1A-1 in promoting cell death involves its ability to regulate the actin cytoskeleton.
Objectives

The specific objectives of this research project are to:

1. Determine whether hepatic eEF1A-1 expression is induced in obese mice, in association with ER stress.

2. Determine whether eEF1A-1 responds to fatty acid overload, or the direct induction of oxidative and ER stress in hepatocytes.

3. Assess the localization of eEF1A-1 to the ER and to polymerized actin during lipid overload conditions.

4. Determine whether targeted knockdown of eEF1A-1 expression confers resistance to saturated fatty acid-induced cell death, and whether its elongation function plays a role in the lipotoxic response.

Relevance to Disease

As discussed previously, eEF1A-1 responds to lipid overload and has been identified as a key factor in mediating lipotoxic cell death (Borradaile et al., 2006a). NAFLD associated with obesity is becoming an increasingly prevalent condition and further studies are required to better understand its cause and progression. It is not yet known whether eEF1A-1 plays a role in the hepatocyte lipotoxic response. Understanding whether eEF1A-1 plays a significant role in mediating lipotoxicity in the liver will provide insight into the pathways involved in this complex disease. This study could have implications for developing therapeutic targets to slow the progression of NAFLD.
2.0 MATERIALS AND METHODS
2.1 Mice

Studies were performed in accordance with the Canadian Guide of the Care and Use of Laboratory Animals and were approved by the Western University Animal Care Committee. Five-week old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were fed ad libitum a chow diet (4% calories from fat, TD8604: Harlan Teklad, Madison, WI) or a high fat Western diet (42% of calories from fat plus cholesterol (0.05% wt/wt), TD96125; Harlan Teklad) for 16 weeks. Five week old male 129SvEv were fed ad libitum a chow diet or high fat Western diet for 15 weeks as described above. Six week old male C57BL/6J mice and leptin-deficient (ob/ob) mice were maintained on AIN-76A Semi-Purified Diet (Harlan Teklad) for 4 weeks. Food intake was measured daily and body weight was measured biweekly. Plasma triglycerides and total cholesterol were determined by enzymatic, colorimetric assays with reagents obtained from Roche Diagnostics. Blood glucose was determined using an Ascensia Elite glucometer (Bayer Healthcare, Toronto, Canada). Plasma insulin was measured using ultrasensitive mouse-specific enzyme-linked immunosorbent assays (ELISA) (Alpco Diagnostics, Windham, NH). Total liver lipids were extracted from 1.0 g sections of liver that had been obtained at sacrifice and stored at -80 °C. Free cholesterol, cholesterol ester and triglycerides from chloroform extracts of liver tissue were determined by enzymatic, colorimetric assays using reagents obtained from Roche Diagnostics and Wako Diagnostics. All plasma and liver lipid measurements were performed through the Metabolic Phenotyping Laboratory in Robarts Research Institute. For analyses of hepatic protein expression, livers were harvested, snap frozen in liquid nitrogen, and tissue homogenates were prepared in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors. eEF1A-1 and GRP78 levels from 20 µg of tissue homogenates were determined by
immunoblotting as described below. Bands were quantified by densitometry and normalized to actin.

2.2 Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown on 100 mm culture dishes at 37°C and 5% CO2. Cells were maintained in 10 ml of Eagles minimum essential medium (EMEM) (Lonza Biowhittaker) with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and 0.5 µg/ml fungizone (Life Technologies). Cells were split (1:6) on a 7 day cycle using trypsin-EDTA. For experiments, cells were plated in 6-well (35 mm), 12-well (18 mm) or 24-well culture plates (Falcon).

For fatty acid treatments, media was supplemented with 0.25 - 1.0 mM palmitate, oleate or a combination of palmitate and oleate (40:60 ratio). The concentrations of fatty acids used reflect high physiological to pathophysiological concentrations, as would be observed during obesity, metabolic syndrome, and type 2 diabetes. The 40:60 ratio of palmitate to oleate is reflective of the saturated to unsaturated fatty acid composition of North American (Westernized) diets, and is maintained in circulating postprandial lipoprotein compositions (Gordon, 1960; Soriguer et al., 2009) For preparation of fatty acids, 20 mM solution of either palmitate or oleate in 0.01 M NaOH was heated to 70°C for 30 min. 1 N NaOH was added dropwise to facilitate solubilization. Fatty acids were complexed to 30% fatty acid free bovine serum albumin (BSA) (Sigma) at a fatty acid to BSA ratio of 2:1. The complexed fatty acids were added to cell culture media to achieve a fatty acid concentration of 0.25 - 1 mM. BSA supplemented medium was used for control conditions.
2.3 Immunoblot Analyses

Whole cell lysates were prepared using RIPA buffer (150 mM NaCl, 1.0% IGEPAL®
CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) (Sigma)
supplemented with protease inhibitors and total cellular protein was quantified using the
Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL).
Lysates were centrifuged for 2 minutes at 14 000 rpm (20 800 g_{av}) to eliminate insoluble
material. Ten µg of whole cell lysate protein was diluted 1:1 with 2X Laemmli sample buffer
(LSB) containing β- mercaptoethanol and heated to 100°C for 5 minutes. Samples were
resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a
0.45 µM nitrocellulose membrane using a semi-dry transfer apparatus. Membranes were incubated
in 1% blocking buffer for 1 h. eEF1A-1 was detected following an overnight incubation at 4°C
with a 1:7500 dilution of anti-eEF1A-1 mouse monoclonal antibody (Cell Signaling
Technology), and a 1 h incubation with a 1:5000 dilution of a horseradish peroxidase (HRP)-
conjugated polyclonal anti-mouse antibody (Santa Cruz Biotechnology). GRP78 was detected
following an overnight incubation with a 1:1000 dilution of anti-GRP78 rabbit polyclonal
antibody (Sigma) and a 1 h incubation with a 1:5000 dilution of HRP-conjugated polyclonal
anti-rabbit antibody (Santa Cruz Biotechnology). Actin was detected following an overnight
incubation with a 1:2000 dilution of anti-actin rabbit polyclonal antibody (Sigma) and a 1 h
incubation with a polyclonal anti-rabbit HRP secondary antibody, as described above.
Membranes were washed 3 times for 5 min with Tris-buffered saline and 0.1% Tween-20
(TBS-T) following incubation with primary and secondary antibodies. Blots were incubated
with Enhanced Chemilumniscence Reagents (Thermo Scientific, Rockford, IL) for 1 min and
exposed on film. Bands corresponding to actin ran between the 37 and 50 kD reference bands.
of the Precision Plus Protein All Blue Standards (Biorad), bands corresponding to eEF1A-1 ran beside the 50 kD reference band, and bands corresponding to GRP78 ran beside the 75 kD reference band. Bands were quantified by densitometry using Quantity One 1-D Analysis Software (Biorad) and normalized to actin.

2.4 Cell Death

Apoptosis and cell death were assessed by Alexa Fluor 488 annexin V staining and membrane permeability to propidium iodide, respectively, using the Dead Cell Apoptosis Kit (Molecular Probes). Following 48 h incubations with various treatments, cells were washed in cold phosphate-buffered saline (PBS) and harvested by trypsinization. 1X annexin-binding buffer was prepared by adding 1 mL 5X annexin binding buffer to 4 mL deionized water, and 100 µg/mL working solution of propidium iodide (PI) was prepared by diluting 1 mg/mL PI stock solution in 45 µL 1X annexin-binding buffer. Cells were centrifuged and resuspended in 1X annexin-binding buffer at a density of 1 x 10^6 cells/ml. After adding 2.5 µl Alexa Fluor 488 annexin V and 2 µl PI to each 200 µl of cell suspension, cells were incubated at room temperature for 15 minutes in the dark. Samples were analyzed by flow cytometry with quantification of 10^4 cells/sample. Apoptotic cells were defined as annexin V positive and PI negative, indicating an intact plasma membrane. Dead cells were defined as annexin V positive and PI positive or PI positive alone, while live cells were negative for both annexin V and PI. Fluorescence emission was measured at 530 nm and >575 nm using 488 nm excitation.
2.5 Immunofluorescence Confocal Microscopy

HepG2 cells were plated on cover glass slides in 6-well plates and allowed to adhere overnight. Once at 70-80% confluence, cells were incubated for 6 h with growth media containing BSA alone, 1.0 mM palmitate, oleate, or a combination of palmitate and oleate (40:60 ratio). Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100, then blocked for 15 min in 0.2% BSA and 10% horse serum in PBS. Following the blocking step, cells were incubated with anti eEF1A-1 monoclonal antibody (1:100 diluted in blocking buffer) for 1 h, washed 2 times for 5 min in PBS then incubated with a 1:100 dilution of FITC-conjugated secondary antibody 30 min. For visualization of ER, cells were incubated with anti-calnexin monoclonal antibody (1:1000) followed by a 1:2000 dilution of secondary antibody conjugated to Alexa Fluor 546 for 30 min. To visualize lipid droplets, cells were stained with Oil Red O (Sigma Aldrich). Staining solution was prepared fresh on day of use by dissolving 0.2 g of Oil Red O powder into 40 ml isopropanol. Working solution was prepared by mixing 30 ml stock solution with 20 ml PBS and filter sterilizing using a 0.22 µm syringe filter. To visualize polymerized actin (F-actin), cells were incubated with rhodamine phalloidin for 15 minutes followed by 5 minute washes (repeated 3 times) in PBS. All coverslips were mounted onto glass slides with mounting media containing DAPI to visualize nuclei (ProLong Gold Antifade Reagent with DAPI, Molecular Probes). Cells were imaged by confocal laser scanning microscopy (Zeiss LSM 510 Meta Confocal Microscope) at the London Regional Cell and In Vitro Molecular Imaging Facility at Robarts Research Institute, and images were processed with Zeiss LSM 5 Image Browser. Colocalization was quantified through Image J using Pearson’s correlation coefficient ($R_c$), which is a standard statistical
an analysis designed to measure the strength of a linear relationship between two variables, in this case fluorescent intensities from two images (Barlow et al., 2010).

2.6 Subcellular Fractionation

HepG2 cells were plated and grown to confluence in 10 cm dishes and incubated with BSA or palmitate for 6 h as described previously. Cells were harvested in 250 mM sucrose and 10 mM Tris HCl containing protease inhibitors and homogenized using a Dounce homogenizer. The homogenate was centrifuged at 800 x g_{av} for 10 minutes to yield a nuclear pellet and a post-nuclear supernatant. The supernatant was centrifuged at 10 000 x g_{av} for 10 minutes to yield a mitochondrial pellet and a post-mitochondrial supernatant. The post-mitochondrial supernatant was layered over a 1.3 M sucrose cushion and centrifuged at 202 000 x g_{av} for 2.5 hours to yield three distinct fractions: a post-microsomal supernatant representing cytosol, a fraction at the interphase of the sucrose cushion representing smooth microsomes and the Golgi apparatus, and a pellet fraction representing rough microsomes. RIPA buffer containing protease inhibitors was added to the smooth and rough microsomal fractions. eEF1A-1 and GRP78 levels from 25 µg of isolated subcellular fractions were determined by immunoblotting as described above.

2.7 [3H] Leucine Incorporation Assay

HepG2 cells were plated in 24-well plates, grown to 70-80% confluency and treated with increasing concentrations of didemnin B (up to 200 nM) or DMSO as a control, for 48 h. Didemnin B was obtained from the Open Chemical Repository of the Developmental Therapeutics Program at the National Cancer Institute (NIH). Following treatment, 1 µL [3H] leucine was added to 250 µl growth medium per well for a specific activity of 500 kBq/ml.
Cells were incubated at 37°C for 60 min. Cells and culture media (for quantification of synthesis of both non-secreted and secreted proteins) were precipitated with cold 1 M trichloroacetic acid (TCA) for 60 min. A sample for total protein quantification was taken prior to precipitation. Samples were centrifuged at 12 000 rpm for 2 min at 4°C and washed 3 times in 100% ethanol by resuspension and centrifugation. The pellet was solubilized in 1 N NaOH and samples were prepared for scintillation counting. Counts measured represented the amount of [³H] leucine incorporated into newly synthesized protein, and were expressed relative to total protein for each sample.

2.8 MTT Assay

HepG2 cells were plated at 50 000 cells per well in a 96-well plate and treated with increasing concentrations of didemnin B up to 200 nM or DMSO as a control for 48 h. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell-metabolism assay. MTT solution was prepared by dissolving 5 mg MTT/ml in sterile PBS at room temperature and filter sterilizing using a 0.2 µm syringe filter. Following treatment, 10 µl MTT was added to each well and incubated for 3 h at 37°C. Formazan crystals formed by the reduction of MTT were dissolved in extraction buffer consisting of 50 ml dimethylformamide (DMF), 50 ml ddH₂O and 20 g SDS adjusted to pH 7.4 and incubated overnight. Absorbance was quantified spectrophotometrically at a wavelength of 570 nm. The intensity of the coloured product formed is directly proportional to the number of live cells present in each sample.
2.9 Transfections

Human eEF1A-1 cDNA sequence was used to design siRNA template oligonucleotides using the Ambion siRNA Target Finder tool. No commercially available pre-designed RNAi reagents are currently available that specifically target eEF1A-1. Hairpin siRNA template oligonucleotide sequences predicted to reduce eEF1A-1 expression were used to generate pSilencer 2.1-U6 expression vectors (Ambion) that were transfected into HepG2 cells. Transfected cells were selected by growth in 500 µg/ml hygromycin for 7 days. Two target sequences that were predicted to generate knockdown of protein expression were selected, however, following immunoblot analysis only one sequence, 5’-AAGTCTGTAATGAAGTGTTAT-3’, was determined to efficiently reduce eEF1A-1 expression. Cells expressing shRNA targeting this sequence were used for subsequent experiments. A control-shRNA expressing population was generated using scrambled shRNA constructs encoding no known target in which eEF1A-1 expression was not reduced.

2.10 Statistical analysis

Statistical analyses were performed using either Student’s t-Test or a one-way ANOVA followed by Tukey’s post hoc test. Differences in means were considered statistically significant at p < 0.05. Immunoblots were quantified by densitometry using Quantity One 1-D Analysis Software (Biorad) and normalized to actin. GraphPad Prism version 5.0 software was used for all statistical analyses and generation of graphs.
3.0 RESULTS
3.1 eEF1A-1 and GRP78 protein are increased in the liver of some mouse models of obesity with hepatic steatosis

eEF1A-1 has been identified as a key mediator of lipotoxic cell death through a genetic screen in CHO cells (Borradaile et al., 2006a). *In vivo*, eEF1A-1 expression is induced in steatotic myocardium of mice with cardiac-specific lipotoxicity, in association with upregulation of markers of oxidative and ER stress (Borradaile et al., 2006a). In the present study, eEF1A-1 protein is increased in some mouse models of obesity, in association with ER stress. In obese, leptin deficient *ob/ob* mice maintained on AIN-76A diet for 4 weeks, hepatic eEF1A-1 and GRP78 protein increased 1.32 and 2.12-fold, respectively, compared to control C57BL/6J mice (Figure 3.1 A). In these mice, liver triglycerides and plasma insulin were elevated 8.9 and 30-fold, respectively (Table 3.1). In C57BL/6J mice maintained on a Western diet for 16 weeks, a 1.39-fold increase in hepatic GRP78 expression was observed while eEF1A-1 expression remained unchanged (Figure 3.1 B). These data suggest eEF1A-1 expression may increase under conditions of hepatic stress caused by severe steatosis and insulin resistance. Interestingly, eEF1A-1 and GRP78 protein are decreased (17% and 42%, respectively) in the livers of 129SvEv mice maintained on a Western diet for 15 weeks compared to chow-fed control mice (Figure 3.1 C). Unlike the other mouse models of obesity, these mice were not hyperinsulinemic (Table 3.1). Increases in body weight, epididymal fat weight, plasma cholesterol, liver triglyceride and liver cholesterol ester were observed in both genetic and diet-induced models (C57BL/6J and 129SvEv) of obesity (Table 3.1). Together, data from C57BL/6J and 129SvEv mice fed a Western diet, as well as obese, leptin deficient *ob/ob* mice demonstrated positive correlations between eEF1A-1 protein expression with both
Figure 3.1 eEF1A-1 and GRP78 protein are increased in some mouse models of obesity with hepatic steatosis. eEF1A-1 and GRP78 protein were detected in whole tissue homogenates from livers of (A) 6 week old wild type (C57BL/6J) or leptin-deficient (ob/ob) mice maintained on AIN-76A diet for 4 weeks, (B) 5 week old C57BL/6J mice maintained on a chow or western diet for 16 weeks, (C) 5 week old 129SvEv mice maintained on a chow or western diet for 15 weeks. eEF1A-1 and GRP78 protein were detected by immunoblotting and quantified by densitometry for 6 chow and 6 western diet fed mice (for both 129SvEv and C57BL/6J strains) and 8 wild type (C57BL/6J) and 8 ob/ob mice. Data are expressed relative to actin. Representative blots are shown. All data are expressed as means ± SEM, * p < 0.05
A

<table>
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<tr>
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<tr>
<td>eEF1A-1</td>
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<td>GRP78</td>
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Protein / Actin Ratio (Relative Densitometry Units)

* * *

B

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<tr>
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Protein / Actin Ratio (Relative Densitometry Units)

* * *

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<td>GRP78</td>
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Protein / Actin Ratio (Relative Densitometry Units)

* * *
Table 1 Parameters of metabolic disease in 3 mouse models of obesity and hepatic steatosis. Mice were maintained as described for Figure 1. Body weight, epididymal fat weight and liver weight were determined at sacrifice. Blood glucose was measured by glucometer. Plasma and liver lipids were measured enzymatically. Plasma insulin was measured by ELISA. Data are expressed as mean ± SEM, *p < 0.05 for 129SvEv or C57BL/6J mice fed a chow diet compared to western diet and ob/ob mice compared to wild-type C57BL/6J mice.
<table>
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<tr>
<th>Mouse</th>
<th>129SvEv</th>
<th></th>
<th>C57BL/6J</th>
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<th>C57BL/6J</th>
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<th>Ob/ob</th>
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<td>Western</td>
<td>Chow</td>
<td>Western</td>
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<td>Number of mice</td>
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<td>Body weight (g)</td>
<td>28.7 ± 0.7</td>
<td>36.4 ± 0.1*</td>
<td>24.9 ± 0.6</td>
<td>33.6 ± 1.2*</td>
<td>22.7 ± 0.6</td>
<td>44.7 ± 0.8*</td>
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<tr>
<td>Epididymal fat weight (g)</td>
<td>0.58 ± 0.05</td>
<td>1.87 ± 0.07*</td>
<td>0.37 ± 0.03</td>
<td>1.6 ± 0.15*</td>
<td>0.43 ± 0.04</td>
<td>3.29 ± 0.09*</td>
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<tr>
<td>Blood glucose (mmol/l)</td>
<td>4.3 ± 0.3</td>
<td>6.1 ± 0.3*</td>
<td>9.0 ± 0.4</td>
<td>11.2 ± 0.4*</td>
<td>7.5 ± 0.5</td>
<td>8.4 ± 1.1</td>
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<tr>
<td>Plasma triglyceride (mmol/l)</td>
<td>0.75 ± 0.07</td>
<td>0.97 ± 0.07*</td>
<td>0.98 ± 0.11</td>
<td>0.68 ± 0.05*</td>
<td>0.40 ± 0.12</td>
<td>0.30 ± 0.06</td>
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<tr>
<td>Plasma cholesterol (mmol/l)</td>
<td>2.88 ± 0.1</td>
<td>4.94 ± 0.09*</td>
<td>2.34 ± 0.13</td>
<td>4.46 ± 0.19*</td>
<td>2.77 ± 0.62</td>
<td>6.38 ± 0.41*</td>
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<tr>
<td>Plasma insulin (ng/ml)</td>
<td>0.58 ± 0.12</td>
<td>0.96 ± 0.22</td>
<td>0.36 ± 0.09</td>
<td>1.66 ± 0.19*</td>
<td>0.47 ± 0.22</td>
<td>14.26 ± 1.74*</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.024 ± 0.031</td>
<td>1.270 ± 0.028*</td>
<td>1.15 ± 0.03</td>
<td>1.31 ± 0.07</td>
<td>0.96 ± 0.01</td>
<td>3.29 ± 0.09*</td>
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<tr>
<td>Liver triglyceride (mg/g)</td>
<td>27.0 ± 1.7</td>
<td>151.2 ± 15.2*</td>
<td>19.49 ± 3.13</td>
<td>77.78 ± 8.40*</td>
<td>25.04 ± 1.58</td>
<td>223.15 ± 35.08*</td>
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<tr>
<td>Liver cholesterol ester (mg/g)</td>
<td>0.92 ± 0.05</td>
<td>8.95 ± 1.56*</td>
<td>0.69 ± 0.09</td>
<td>3.10 ± 0.43*</td>
<td>1.26 ± 0.28</td>
<td>5.72 ± 0.24*</td>
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<tr>
<td>Liver free cholesterol (mg/g)</td>
<td>3.10 ± 0.11</td>
<td>3.04 ± 0.13</td>
<td>2.43 ± 0.12</td>
<td>2.53 ± 0.11</td>
<td>1.65 ± 0.06</td>
<td>2.31 ± 0.08*</td>
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liver triglyceride (Figure 3.2 A) and plasma insulin (Figure 3.2 B), and between GRP78 expression and plasma insulin (Figure 3.2 D).
Figure 3.2 Hepatic eEF1A-1 expression correlates with liver triglyceride content and plasma insulin concentration. Liver triglycerides and plasma insulin are plotted against GRP78 and eEF1A-1 expression for 3 mouse models of obesity. 5 week old 129SvEv mice were maintained on a chow or western diet (WD) for 15 weeks, 5 week old C57BL/6J mice were maintained on a chow or western diet for 16 weeks and 6 week old wild type (C57BL/6J) or leptin-deficient (*ob/ob*) mice were maintained on AIN-76A diet for 4 weeks. Triglycerides from chloroform extracts of liver tissue were quantified enzymatically, and plasma insulin was quantified by ELISA. eEF1A-1 and GRP78 protein were detected by immunoblotting and quantified by densitometry for 6 chow and 6 western diet fed mice (for both 129SvEv and C57B/6 strains) and 8 wild type (C57BL/6J) and 8 *ob/ob* mice. Protein are expressed relative to actin. Significant correlations (p < 0.05) are indicated with $r^2$ value.
A

B

C

D

C57BL/6J AIN76A
Ob/ob AIN76A
129SvEv Chow
129SvEv WD
C57BL/6J Chow
C57BL/6J WD
Ob/ob AIN76A

r² = 0.1447

r² = 0.4558

r² = 0.3536
3.2 eEF1A-1 protein is rapidly induced in response to saturated fatty acid overload-induced ER stress in HepG2 cells, an event that precedes cell death

Having determined that eEF1A-1 is increased under conditions of severe hepatic steatosis and insulin resistance in mice, we set out to characterize and modulate expression levels of eEF1A-1 in an in vitro model to determine the role of this protein in the hepatocyte lipotoxic response. Previous studies showed that levels of eEF1A-1 increase in CHO cells and H9c2 rat cardiomyocytes in response to palmitate (Borradaile et al., 2006a). In these cells, eEF1A-1 expression was rapidly induced (30 minutes and 1 h, respectively) and remained elevated for 5 h after treatment with palmitate (Borradaile et al., 2006a). For the present study, we selected HepG2 human hepatoma cells as an in vitro model of hepatocytes. HepG2 cells are well suited for this application because, like human and rodent liver, and unlike other hepatocyte lines, they exclusively express the eEF1A-1 variant of eEF1A (Grassi et al., 2007; Newbery et al., 2007). In HepG2 cells incubated for 6 h with growth media containing either palmitate (1.0 mM) or a combination of palmitate and oleate (1.0 mM, 40:60 ratio), eEF1A-1 protein increased 1.24-fold and 1.72-fold, respectively. In contrast, incubation with 1.0 mM oleate decreased eEF1A-1 expression by 16% (Figure 3.3 A). Changes in eEF1A-1 content were accompanied by similar changes in GRP78 content in palmitate treated cells, indicating the onset of ER stress under these lipid overload conditions (Figure 3.3 B).

In addition to fatty acid induced cell stress, eEF1A-1 has been shown to be directly induced in response to oxidative and ER stress (Chen et al., 2000; Borradaile et al., 2006a). For the present study, H₂O₂ was used to induce oxidative stress and tunicamycin was used to induce ER stress. Tunicamycin inhibits protein glycosylation and subsequent protein folding, and is widely used as an inducer of ER stress and the UPR (Lee, 2001). An increasing trend in
Figure 3.3 eEF1A-1 expression is increased in HepG2 cells during fatty acid overload induced ER stress. HepG2 cells were incubated for 6 h with growth media containing BSA alone, palmitate (PA), oleate (OA) or a combination of palmitate and oleate (40:60 ratio) at concentrations of 0.25, 0.5 and 1.0 mM. All fatty acids were conjugated to BSA at a molar ratio of 2:1. (A) eEF1A-1 and (B) GRP78 protein were detected in whole cell lysates by immunoblotting. All data were quantified by densitometry and normalized to actin. Representative blots are shown beside each graph. Fatty acid treatments are compared to the lowest respective concentration (0.25 mM). Data are expressed as mean ± SEM for 3-6 independent experiments, *p < 0.05.
A

**eEF1A-1/Actin Ratio**

- **Concentration (mM)**
  - BSA
  - PA
  - OA
  - PA:OA

B

**GRP78/Actin Ratio**

- **Concentration (mM)**
  - BSA
  - PA
  - OA
  - PA:OA
eEF1A-1 expression was noted following incubation with 1.0 µg/ml tunicamycin for 6 h. In addition, eEF1A-1 protein increased 1.97-fold in cells treated with 2.5 mM H₂O₂, indicating that eEF1A-1 expression in hepatocytes also responds to direct induction of oxidative stress (Figure 3.4 A). GRP78 expression increased 2.41-fold following treatment with tunicamycin (Figure 3.4 B).

Saturated fatty acids are known to trigger cellular stress responses leading to apoptotic cell death in hepatocyte cell lines and primary hepatocytes, as well as in other cell types (Wei et al., 2006; Li et al., 2009). Consistent with these earlier studies, treatment of HepG2 cells with 1.0 mM palmitate for 48 h resulted in a 2.24 fold increase in the proportion of cell death compared to BSA treated cells (Figure 3.5). No difference in cell death was observed in oleate, or a combination of palmitate and oleate treated cells.
Figure 3.4 eEF1A-1 expression is increased in HepG2 cells in response to direct induction of oxidative or ER stress. HepG2 cells were incubated for 6 h with 2.5 mM H$_2$O$_2$ or 1.0 µg/ml tunicamycin. Control cells were incubated for 6 h with water or DMSO, respectively. (A) eEF1A-1 expression and (B) GRP78 expression were detected at the protein level in whole cell lysates by immunoblotting. All data were quantified by densitometry and normalized to actin. Representative blots are shown below each graph. Tunicamycin and H$_2$O$_2$ treated cells are compared to control. Data are expressed as mean ± SEM for 3-6 independent experiments, *p < 0.05.
A) eEF1A-1

B) GRP78

Graphs showing the relative densitometry units for eEF1A-1 and GRP78 under control and stress conditions with and without Tunicamycin and H2O2 treatments.
Figure 3.5 Palmitate induces lipotoxicity in HepG2 cells.

Confluent HepG2 cells were incubated for 48 h with growth media containing BSA alone, 1.0 mM PA, OA or a combination of PA and OA (40:60 ratio). All fatty acids were conjugated to BSA at a molar ratio of 2:1. Cells were harvested, stained with propidium iodide, and the proportion of dead cells was determined by flow cytometry. Fatty acid treatments are compared to BSA control. All data are expressed as mean ± SEM for 4-8 independent experiments, *p < 0.05.
Total Cell Death (% of Population)

- BSA
- PA
- OA
- PA:OA
3.3 Palmitate induces eEF1A-1 colocalization with the actin cytoskeleton

In addition to its canonical role in peptide elongation, eEF1A-1 is known to participate in remodeling of the actin cytoskeleton, which undergoes dramatic changes during apoptosis and cell death (Murray et al., 1996; Ndozangue-Touriguine et al., 2008). eEF1A-1 has also been reported to associate with lipid droplets and the ER in various cell types (Zhang et al., 2011; Bouchoux et al., 2011; Chen et al., 2010; Minella et al., 1996). To assess the localization of eEF1A-1 with the actin cytoskeleton, as well as its association with lipid droplets and the ER in response to lipid overload, HepG2 cells were treated for 6 h with fatty acids, fixed, stained, and imaged by confocal fluorescent microscopy, as described in Materials and Methods.

eEF1A-1 has been identified to associate with lipid droplets in proteomic analyses of neutral lipid droplets from mouse skeletal muscle and intestinal enterocytes (Zhang et al., 2011; Bouchoux et al., 2011). However, our experiments indicated no colocalization of eEF1A-1 with cytosolic neutral lipid droplets in HepG2 cells in either of the fatty acid treatment conditions (Figure 3.6 C-F). As seen in Figure 3.6 G-H, enlargement of the outlined area indicates that eEF1A-1 surrounds lipid droplets but does not colocalize with or decorate these neutral lipid stores.

eEF1A-1 has been found predominantly localized to the ER in fibroblast cells, particularly in association with ribosomes (Hayashi et al., 1989). Consistent with these studies, our confocal fluorescent microscopy images indicate that eEF1A-1 colocalizes with the ER in hepatocytes under basal conditions (Figure 3.7 C-F). Colocalization was quantified through ImageJ using Pearson’s correlation coefficient (R), in order to measure the strength of the
**Figure 3.6 eEF1A-1 does not colocalize with lipid droplets in HepG2 cells.** HepG2 cells were incubated for 6 h with growth media containing BSA alone, 1.0 mM PA, OA or a combination of PA and OA (40:60 ratio). All fatty acids were conjugated to BSA at a molar ratio of 2:1. eEF1A-1 and lipid droplets were assessed by confocal fluorescent microscopy. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with anti-eEF1A monoclonal antibody followed by secondary conjugated to FITC (green) to visualize EF1A-1 (A). Cells were stained with Oil Red O (ORO) to visualize neutral lipids (red) (B) and counterstained with DAPI to visualize nuclei (blue). Localization of eEF1A-1 with lipid droplets was assessed in cells treated with BSA (C), PA (D), OA (E) and a combination of PA and OA (F). Enlargement of the outlined area in (F) indicate that no colocalization was observed between eEF1A-1 and cytosolic neutral lipid droplets. Representative images from 3 independent experiments are shown.
Figure 3.7 eEF1A-1 colocalization with the ER is decreased during exposure to excess palmitate. HepG2 cells were incubated for 6 h with growth media containing BSA alone, palmitate (PA), oleate (OA) or a combination of palmitate and oleate (40:60 ratio) at concentrations of 0.25, 0.5 and 1.0 mM. All fatty acids were conjugated to BSA at a molar ratio of 2:1. eEF1A-1 and ER were assessed by confocal fluorescent microscopy. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with anti-eEF1A monoclonal antibody followed by secondary conjugated to FITC (green) to visualize EF1A-1 (A). Cells were incubated with anti-calnexin monoclonal antibody followed by secondary conjugated to Alexa Fluor 546 (red) to visualize ER (B) and counterstained with DAPI to visualize nuclei (blue). Colocalization of eEF1A-1 with ER was assessed in cells treated with BSA (C), PA (D), OA (E) and a combination of PA and OA (40:60 ratio) (F). Colocalized signal is seen in yellow and was quantified using Pearson’s correlation coefficient ($R_p$) to assess overlap between signal for eEF1A-1 and calnexin (G). (H) eEF1A-1 protein levels were detected in cytosolic, smooth microsomal, and rough microsomal fractions isolated by sequential centrifugations in the absence or presence of palmitate (8 h treatment). GRP78 and actin were detected to determine the relative enrichment and contamination of each fraction. Representative blots are shown. All data are expressed as mean ± SEM for 3 independent experiments, *p < 0.05.
relationship between fluorescent intensities from two images (Barlow et al., 2010). This value ranges from -1.0 to 1.0, where 0 indicates random distribution, -1.0 indicates complete inverse correlation and 1.0 indicates complete positive correlation between fluorescent signals (Zinchuk and Grossenbacher-Zinchuk, 2009). In our experiments, the \( R_r \) for eEF1A-1 colocalization with F-actin or ER ranged from -0.0023 to 0.60 in all treatments tested. In HepG2 cells incubated for 6 h in the presence of palmitate, a 30% decrease in colocalization of eEF1A-1 with the ER was observed compared to BSA treated cells (Figure 3.7 G). Subcellular fractionation of HepG2 cells confirmed that eEF1A-1 is enriched in ER fractions (Figure 3.7 H).

The decrease in colocalization of eEF1A-1 with the ER in response to excess palmitate was accompanied by a corresponding increase (2.79-fold) in colocalization of eEF1A-1 with the actin cytoskeleton (Figure 3.8). Images constructed from a z-series of optical sections indicated that under basal conditions, F-actin was primarily localized to the basolateral surface of the cell monolayer while eEF1A-1 was localized apically (Figure 3.8 G). Treatment with palmitate resulted in increased colocalized signal at the basolateral surface (Figure 3.8 H).

3.4 Inhibition of eEFA-1 elongation function decreases palmitate-induced cell death

To determine whether the peptide elongation function of eEF1A-1 plays a role in the lipotoxic response, HepG2 cells were incubated with didemnin B, a member of a class of cyclic depsipeptides produced by ascidians (marine tunicates) that are known to have eEF1A-1-dependent inhibitory effects on protein synthesis (Marco et al., 2003). Didemnin B specifically binds GTP-bound eEF1A-1 at the ribosome and inhibits its release from the ribosomal A-site, thus attenuating protein synthesis (Marco et al., 2003). HepG2 cells were treated for 48 h with increasing concentrations of didemnin B, followed by assessment of total
Figure 3.8 eEF1A-1 colocalization with the actin cytoskeleton is increased during exposure to excess palmitate. HepG2 cells were incubated for 6 h with growth media containing 1.0 mM palmitate PA conjugated to BSA (molar ratio 2:1), OA conjugated to BSA (molar ratio 2:1), a combination of palmitate and oleate (40:60 ratio), or BSA alone. eEF1A-1 and polymerized actin, and were assessed by confocal fluorescent microscopy. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with anti-eEF1A monoclonal antibody for 1 h and secondary conjugated to FITC (green) for 30 min to visualize EF1A-1 (A). Cells were stained with rhodamine phalloidin to visualize F-actin (red) (B) and counterstained with DAPI to visualize nuclei (blue). Colocalization of eEF1A-1 with ER was assessed in cells treated with BSA (C), PA (D), OA (E) and a combination of PA and OA (40:60 ratio) (F). (G) and (H) show images constructed from a z-series of optical sections of cells treated with BSA and PA, respectively. Colocalized signal is seen in yellow and was quantified using Pearson’s correlation coefficient (R_p) to assess overlap between signal for eEF1A-1 and phalloidin (I). All data are expressed as mean ± SEM 4 independent experiments, *p < 0.05.
**Figure I**

- **Panel A** shows an image labeled with "10 μM".
- **Panel B** displays a similar image.
- **Panel C** features another labeled image.
- **Panel D** presents an image with a different label.
- **Panel E** includes an image with the label "10 μM".
- **Panel F** shows an image with a respective label.

**Graph (I)** represents the eEF1A-1/Phalloidin Rt Value with bars for BSA, PA, OA, and PA:OA treatments. The error bars and asterisk (*P < 0.05) denote statistical significance.
protein synthesis by $[^3]H$ leucine incorporation over 1 h (Figure 3.9 A). Concentrations used were selected based on previous studies of didemnin B in human cancer cell lines (Beidler et al., 1999). The IC$_{50}$ for inhibition of protein synthesis was determined to be 80 nM (Figure 3.9 A). Cell viability, measured using the MTT cell-metabolism assay, was not affected at this IC$_{50}$ concentration (Figure 3.9 B). In subsequent experiments, cells were treated with 1.0 mM palmitate in the presence or absence of 80 nM didemnin B for 48 h followed by assessment of cell death by flow cytometry. Treatment with palmitate in the presence of didemnin B decreased palmitate-induced cell death (Figure 3.9 C), suggesting that the elongation function of eEF1A-1 may play a role in the lipotoxic response.

### 3.5 Modest long-term inhibition of eEF1A-1 expression alters HepG2 morphology

Previous studies showed that knockdown of eEF1A-1 expression in CHO cells and H9c2 cardiomyocytes conferred resistance to palmitate-induced cell death (Borradaile et al., 2006a). To extend these findings to hepatocytes, a cell type that, similar to cardiomyocytes, is affected by lipotoxicity in metabolic disease, we generated two populations of HepG2 cells with stable expression of shRNA predicted to target eEF1A-1 message. Control cells expressed an shRNA construct encoding no known target. Upon assessment of the extent of eEF1A-1 knockdown by immunoblotting, only one population (EF shRNA) was identified as having significantly reduced eEF1A-1 expression (18%) (Figure 3.10 A). This population was used for subsequent experiments. Although the reduction of eEF1A-1 expression in EF shRNA expressing cells is modest, this protein is abundant in cells and represents about 2% of total cellular protein (Ejiri et al., 2002). Thus small decreases in relative levels of eEF1A-1 reflect large decreases in total protein production. Unlike in H9c2 cardiomyocytes,
Figure 3.9 Inhibition of eEF1A-1 elongation function decreases palmitate-induced cell death. HepG2 cells were treated for 48 h with increasing concentrations of didemnin B (inhibitor of eEF1A-1 elongation function), or DMSO as a control, followed by assessment of total protein synthesis by $[^3H]$ leucine incorporation. The $IC_{50}$ for inhibition of protein synthesis was 80 nM. (B) Cells were incubated as described above, followed by incubation for 3 h with 5 mg/ml MTT. MTT reduction, indicative of cell viability, was quantified spectrophotometrically at a wavelength of 570 nm. (C) HepG2 cells were incubated for 48 h with growth media containing 1.0 mM PA conjugated to BSA (molar ratio 2:1) in the presence or absence of 80 nM didemnin B ($IC_{50}$). Cells were harvested, stained, and the proportions of live cells (Ann V$^-\text{PI}^-$), apoptotic cells (Ann V$^+\text{PI}^-$) and dead cells (Ann V$^+\text{PI}^+$) were determined by flow cytometry. For A and B, data are expressed as percentage of control ± SEM for 3 independent experiments. For C, data are expressed as mean ± SEM for 4 independent experiments, *p < 0.05 for PA compared to BSA control.
**A**

[^3]H Leucine Incorporation

% of Control

\[
\text{log} \ [\text{didemnin B (nM)}]
\]

**B**

MTT Reduction Abs\(_{570}\)

% of Control

Didemnin B (nM)

**C**

% of Population

BSA  Did B  PA  PA + Did B

- Live Cells
- Apoptotic Cells
- Dead Cells

* Statistically significant difference.
Figure 3.10 Modest long-term inhibition of eEF1A-1 expression alters HepG2 morphology. HepG2 cell lines were generated expressing either control shRNA against no known target, or shRNA targeted against eEF1A-1 (EF shRNA) (A). eEF1A-1 protein levels from whole cell lysates were detected by immunoblotting. All data were quantified by densitometry and normalized to actin. A representative blot from 5 independent experiments is shown (A). (B) Control shRNA and EF shRNA expressing cells were incubated for 48 h with growth media containing 1.0 mM PA conjugated to BSA (molar ratio 2:1) or BSA alone. Cells were harvested, stained with ann V and PI, and the proportions of live cells (Ann V− PI−), apoptotic cells (Ann V+ PI−) and dead cells (Ann V+ PI+) were determined by flow cytometry. PA treated cells are compared to BSA for both control and EF shRNA expressing cells. Data are expressed as mean ± SEM for 3 independent experiments, *p < 0.05. (C) Cell morphology of control shRNA or EF shRNA expressing cells and DMSO or didemnin B treated wild-type HepG2 cells.
A

![Image of Western Blot](image1)

B

![Bar Chart](image2)

C

![Microscopy Images](image3)
knockdown of eEF1A-1 expression was not accompanied by resistance to palmitate-induced cell death (Figure 3.10 B). However, EF shRNA expressing cells exhibited altered cell morphology compared to control shRNA expressing cells (Figure 3.10 C). EF shRNA expressing cells exhibited elongated projections extending from the cell body and enlarged spherical structures, resembling vacuoles, within the cell body. This altered morphology was not evident in wild-type HepG2 cells treated with didemnin B, thus these morphological changes are not likely due to inhibition of the canonical elongation function of eEF1A-1 alone.

Taken together this data suggests that eEF1A-1 expression is induced in the livers of obese mice as well as in HepG2 human hepatoma cells in response to excess palmitate, and may participate in the process of lipotoxicity by mediating changes in protein synthesis related to actin cytoskeleton remodeling that occur during cell stress.
4.0 DISCUSSION
4.1 Summary of Results

As much as 30% of the general population is currently affected with NAFLD, while the prevalence in the morbidly obese is estimated at 75-92%, making this condition one of the most common liver disorders worldwide (Trauner et al., 2010; Erickson, 2009). However, the cause and progression of NAFLD is complex and not fully understood. Based on previous findings identifying eEF1A-1 as a mediator of lipotoxic cell death in CHO cells and cardiomyocytes, a similar role for this protein in hepatocytes was proposed. We hypothesized that eEF1A-1 protein responds to lipid overload in hepatocytes and in the steatotic livers of obese mice, and participates in the process of lipotoxicity. Furthermore, we proposed that the mechanism of action of eEF1A-1 in lipotoxicity may involve its ability to regulate the actin cytoskeleton. Our studies have revealed several key findings. 1) eEF1A-1 protein is induced, in association with ER stress, in a mouse model of obesity with severe hepatic steatosis, and in cultured hepatocytes exposed to excess saturated fatty acids. 2) A consequence of palmitate overload in hepatocytes is increased colocalization of eEF1A-1 with the actin cytoskeleton and decreased colocalization with the ER, followed by cell death, suggesting eEF1A-1 may regulate changes in the actin cytoskeleton required for the progression of cell death. 3) Inhibition of the elongation function of eEF1A-1 diminishes palmitate induced cell death, while modest long-term inhibition of eEF1A-1 expression alters hepatocyte morphology with no effect on palmitate-induced cell death, suggesting that the canonical function of eEF1A-1 may play a role in lipotoxicity.
4.2 eEF1A-1 in mouse models of obesity and hepatic steatosis

Our data from diet induced and genetic models of obesity indicate that eEF1A-1 protein content is increased under conditions of hepatic stress associated with severe steatosis and insulin resistance. In addition to findings that eEF1A-1 protein is increased in steatotic mouse myocardium (Borradaile et al., 2006a), changes in its expression have been reported in several other obese and diabetic models. In an analysis of the dysregulated genes associated with chronic hepatic steatosis, eEF1A-1 expression was increased in hepatocytes isolated from obese insulin-resistant Zucker rats compared to lean littermates (Buqué et al., 2009). Furthermore, others have shown that EF1A-1 expression is increased in the skeletal muscle of both type 1 and type 2 diabetic patients, in skeletal muscle of streptozotocin-induced diabetic rats (Reynet and Kahn, 2001), and in the renal cortex of diabetic rats (Al-Maghrebi et al., 2005). These previous findings, along with our own observations that eEF1A-1 protein expression is positively correlated with both liver triglyceride and plasma insulin, suggest eEF1A-1 may play a role in the cellular pathways responsible for the pathogenesis of metabolic syndrome.

4.3 eEF1A-1 as a response protein during saturated fatty acid induced ER stress and cell death

In the present study, we show that eEF1A-1 responds to palmitate overload in hepatocytes, consistent with previous findings in CHO cells and cardiomyocytes (Borradaile et al., 2006a). Palmitate is the most common saturated fatty acid in our diet and cells were treated with concentrations of fatty acids (up to 1.0 mM) that mimic those encountered in pathophysiological conditions. We further found that eEF1A-1 protein was induced in cells
treated with a combination of palmitate and oleate (40:60 molar ratio), which has not been reported previously in any cell type. Of note, treatment with palmitate plus oleate was not severe enough to induce ER stress or cell death, as was seen in palmitate treated HepG2 cells. However, this combination is most similar to the fatty acid composition of North American (Westernized) diets suggesting these results may have implications in vivo.

4.4 Subcellular localization of eEF1A-1 during fatty acid overload

eEF1A-1 is known to bind to and regulate polymerization of the actin cytoskeleton (Shiina et al., 1994; Murray et al., 1996). Because the cytoskeleton undergoes dramatic changes during apoptosis and cell death, we wanted to determine the subcellular localization of eEF1A-1 under basal and lipid overload conditions in hepatocytes. Our initial findings indicated relatively low colocalization of eEF1A-1 with the actin cytoskeleton under basal conditions. Our images constructed from a z-series of optical sections indicate that, under basal conditions, actin is primarily localized to the basolateral surface of the cell monolayer, while eEF1A-1 is localized apically. These observations likely explain our difficulty in acquiring confocal microscopy images in which the two proteins are present in the same optical plane. Our finding that palmitate treatment increased colocalization of eEF1A-1 with the actin cytoskeleton, combined with previous evidence that palmitate treatment can increase polymerized actin content (Borradaile et al., 2006a) suggest that the response of eEF1A-1 to saturated fatty acids in hepatocytes may involve regulation of cytoskeletal dynamics by promoting actin polymerization. Similar effects on actin distribution are seen in cells treated with H$_2$O$_2$, preceding cell death (Dalle-Donne et al., 2001; Zhu et al., 2005), conditions which we showed also induce eEF1A-1 protein. Our finding that EF1A-1 localizes to the actin
cytoskeleton and toward the basolateral surface of the cell during palmitate-induced stress may also indicate increased localization at cell membrane surfaces that are in contact with the surface of the cell culture plate. This is consistent with recent evidence indicating that eEF1A-1 plays a role in anoikis through increased localization to the cell membrane (Itagaki et al., 2012).

Others have reported changes in the localization of eEF1A-1 to actin in response to external stimuli. Dharmawardhane et al. (1991) observed an increased incorporation of eEF1A-1 into the actin cytoskeleton upon stimulation with the chemoattractant cAMP in the actin-filament containing regions of the cell cortex and in the filopodia (Dharmawardhane et al., 1991). However, this response was not involved in promoting cell death, as is likely the case in our HepG2 cell experiments, but instead was involved with the extension of cell surface projections.

Our finding that eEF1A-1 is mainly localized in the cytoplasmic compartment of hepatocytes and colocalizes to the ER under basal conditions is consistent with findings in other cell types (Minella et al., 1996; Chen et al., 2010). We further show here for the first time that a decrease in colocalization of eEF1A-1 with the ER occurs following treatment with palmitate. Taken together, these data, along with our results that significant palmitate-induced cell death does not occur until 48 h, indicate that eEF1A-1 may mediate palmitate-induced cell death by translocation toward the actin cytoskeleton to regulate changes in actin polymerization required for the progression of cell death.

Although recent evidence from proteomic analyses of cytosolic lipid droplets suggests eEF1A-1 associates with lipid droplets in various metabolic tissue cell types (Bartz et al.,...
2007; Zhang et al., 2011; Bouchoux et al., 2011; Ding et al., 2012), our findings do not support these conclusions. Our confocal microscopy work indicates that eEF1A-1 does not associate with or decorate cytosolic lipid droplets, as is seen with other lipid droplet-associated proteins such as perilipin (Buers et al., 2011). This suggests that the association of eEF1A-1 with lipid droplets reported in previous studies may be found in specific cellular components, such as the ER, that were co-purified with the lipid droplet fraction. Lipid droplet formation originates at the ER, where it is believed that neutral lipids accumulate to form a lens between the two leaflets of the phospholipid bilayer (Brasaemle and Wolins, 2012). The fact that eEF1A-1 is highly localized to the ER makes it likely that biochemical isolation of lipid droplets for proteomic analyses results in a preparation that is contaminated with some ER localized proteins, including eEF1A-1.

4.5 Inhibition of eEF1A-1 elongation function and response to lipotoxicity

Although the peptide elongation function of eEF1A-1 is well characterized, it has not been previously implicated in lipotoxicity. Didemnin B is a naturally occurring cyclic peptide that inhibits protein synthesis by binding GTP-bound eEF1A-1 at the ribosome and preventing ribosomal translocation (Ahuja et al., 2000; Marco et al., 2004). Thus, it specifically inhibits the elongation function of eEF1A-1. Since its discovery in 1981, didemnin B has been to show to have various antitumor, antiviral and immunosuppressive functions and was the first compound isolated from a marine source to go into clinical trials for human diseases (Lee et al, 2012).

Work from several laboratories has suggested that accumulation of unfolded proteins in the ER and activation of the UPR itself is sufficient to cause hepatic steatosis (Imrie and
Sadler, 2012). This suggests a possible disease scenario in which increased fatty acid flux from adipose tissue to the liver initially induces ER stress, which in turn induces hepatic de novo lipogenesis, which subsequently worsens hepatic ER stress, leading to the progression of NAFLD. Our results show that treatment with palmitate in the presence of didemnin B decreases palmitate-induced cell death, suggesting that the elongation function of eEF1A-1 may play a role in the lipotoxic response. Thus, it is possible that inhibition of protein synthesis during palmitate overload may decrease the burden on the ER, which may prevent activation of the UPR, ER stress response and subsequent activation of downstream apoptotic pathways. This concept is supported by recent evidence that suggests alleviating ER stress and the accumulation of misfolded proteins may serve as a possible therapeutic strategy for the treatment NAFLD (Ibrahim et al., 2011). Small chemical chaperones such as glycerol or 4-phenyl butyric acid (PBA) improve ER stress by increasing ER protein folding capacity and facilitate the appropriate trafficking of misfolded proteins (Clark and Diehl, 2003). Treatment of obese and diabetic mice with these compounds reduces ER stress, decreases hepatic steatosis and improves liver function enzyme levels (Ozcan et al., 2006).

4.6 Knockdown of eEF1A-1 in hepatocytes and response to lipotoxicity

To determine whether knocking down eEF1A-1 expression could protect hepatocytes from palmitate-induced cell death, we generated a stable population of HepG2 cells in which eEF1A-1 expression was modestly reduced. We did not find that hepatocytes with reduced eEF1A-1 expression were protected from lipotoxic cell death. This may be explained by the limited extent of knockdown that was achieved (18% reduction). However, it is also possible
that other, protective or adaptive functions of eEF1A-1 may be disrupted by reducing its expression – functions which may not be disrupted by didemnin B.

eEF1A-1 is highly expressed in the liver and in other tissues with high rates of protein synthesis and cell proliferation (lung, kidney, pancreas, placenta) (Knudsen et al., 1993). Although eEF1A-1 is not rate-limiting for protein synthesis, it has roles in many other cellular processes (Mateyak and Kinzy, 2010), therefore it is possible that hepatocytes with a greater reduction of eEF1A-1 were not viable for extended periods of time. Although no changes in the cell death response to palmitate were observed in our EF shRNA expressing HepG2 population were observed, these cells exhibited an altered phenotype compared to control shRNA expressing cells. Interestingly, Gross and Kinzy (2005) noted a difference in morphology of cells in which the interaction between eEF1A-1 and actin was disrupted. Yeast with mutations in the eEF1A-1 domain responsible for actin binding were shown to be larger in size and exhibited pronounced elongated buds (Gross and Kinzy, 2005). Thus, it is possible that even a small reduction in total protein expression may be sufficient to disrupt eEF1A-1-actin interactions and result in altered cytoskeletal organization.
Figure 4.1 Working model for the role of eEF1A-1 in hepatocyte response to saturated fatty excess. During exposure to excess saturated fatty acids, cellular capacities to store them as triglycerides (lipid droplets), to catabolize them through β-oxidation (mitochondria), or secrete them in the form of VLDL particles are overwhelmed. Under basal conditions, the majority of eEF1A-1 is localized to the ER and little is associated with the actin cytoskeleton. Palmitate overload leads to mobilization of eEF1A-1 away from the ER and toward the actin cytoskeleton to mediate changes in its structure, likely by promoting actin polymerization and potentially actin synthesis, for the progression of cell death.
4.7 Future Directions

Our working model for the role that eEF1A-1 plays in the stress response to saturated fatty acid excess in hepatocytes is summarized in Figure 4.1. Based on this model, and our evidence of increased eEF1A-1 protein in the livers of obese, leptin-deficient mice with severe hepatic steatosis (ob/ob) mice, several future experiments are warranted.

To further support our evidence of the interaction between eEF1A-1 and the actin cytoskeleton during palmitate overload, I would like to determine if eE1A-1 functions by regulating cytoskeleton dynamics, as is seen in other cell types. To do so, I would disrupt the interaction of eEF1A-1 with the actin cytoskeleton by transfecting cells with a construct encoding domain III of eEF1A-1 (the dominant actin-binding domain). This construct is expected to have a dominant-negative action on the eEF1A-1-actin interaction. To induce lipid overload and cell death, cells will be treated with palmitate and cell death will be assessed by flow cytometry.

Based on our results that treatment with didemnin B reduces palmitate-induced cell death in HepG2 cells, future studies should be directed towards testing the effectiveness of didemnin B in improving hepatocyte damage in mouse models of NAFLD. The results presented in this thesis indicate that eEF1A-1 expression is increased in the livers of leptin deficient ob/ob mice and these animals exhibited the most severe form of hepatic steatosis and ER stress. Furthermore, ob/ob mice are a well-characterized model of NAFLD (Anstee and Goldin, 2006). Thus, I would select this model for subsequent didemnin B experiments. Previous work has demonstrated that didemnin B is highly concentrated in the livers of mice following intraperitoneal administration (Beasley et al., 2005). To determine if ER stress and
hepatocyte damage is attenuated in the livers of these mice treated with didemnin B, liver samples will be analyzed for GR78 expression, PERK phosphorylation and JNK activity. These markers of ER stress are consistently exhibited in ob/ob mice (Ozcan et al., 2004). Serum measurements of ALT and AST activity will be used as additional indicators of liver damage.

The liver is composed of hepatocytes and non-parenchymal cells, which include kupffer cells, sinusoidal endothelial cells and hepatic stellate cells (Bian and Ma, 2012). Hepatic stellate cells play a key role in the hepatic fibrogenesis associated with NASH. Under normal conditions, hepatic stellate cells are maintained in a quiescent state but can undergo a phenotypic transition to become activated in response to liver injury. These cells are the major source of the excessive extracellular matrix production that characterizes fibrotic liver (Viera and Nato, 2006). It is possible that eEF1A-1 may play a role in the progression to fibrosis during the process of hepatic stellate cell activation. Future experiments in which expression of eEF1A-1 in hepatic stellate cells is reduced in obese mice may give us further insight into the role this protein plays in the progression of NAFLD.
5.0 REFERENCES


Fate of tritiated didemnin B in mice: excretion and tissue concentrations after an intraperitoneal dose. Biopharm Drug Dispos 26:341-351.


APPENDIX A

Animal Use Protocols
February 18, 2010

This is the Original Approval for this protocol
*A Full Protocol submission will be required in 2014*

Dear Dr. Borradaile:

Your Animal Use Protocol form entitled:
Liver Function During Obesity and Type 2 Diabetes
Funding Agency Canadian Lipoprotein Conference

has been approved by the University Council on Animal Care. This approval is valid from February 18, 2010 to February 28, 2011. The protocol number for this project is 2010-018.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
4. If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

ANIMALS APPROVED FOR 4 Years

<table>
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<th>4 Year Total Numbers Estimated as Required</th>
<th>List All Strain(s)</th>
<th>Age / Weight</th>
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<td>240</td>
<td>C57Bl/6/ ob/ob</td>
<td>4-5 weeks</td>
</tr>
</tbody>
</table>

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Approval - N. Borradaile, W. Lagerwerf
Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Vascular Regeneration After Increased Nad+ Availability In Diet Induced Metabolic Syndrome" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care
AUP Number: 2010-018
AUP Title: Liver function during obesity and type 2 diabetes

Yearly Renewal Date: 03/01/2013

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2010-018 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

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PRESENTATIONS & CONFERENCES

Stoianov A, Robson D, Borradaile NM. Palmitate induces elongation factor (eEF) 1A-1 colocalization with the actin cytoskeleton in hepatocytes. Physiology and Pharmacology Research Day, Western University. November 6, 2012. [poster]


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Stoianov A, Borradaile NM. Hepatocyte response to fatty acid overload involves eukaryotic elongation factor (eEF) 1A-1. Physiology and Pharmacology Research Day, Western University. November 8, 2011. [poster]

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