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Master's Thesis/Practicum Final Report

The undersigned certify that they have read the Master's Thesis/Practicum entitled:

Regulation of Cholesterol Biosynthesis in Hepatocytes

submitted by

Jennifer Emily Enns

in partial fulfillment of the requirements for the degree of

Master of Science

The Thesis/Practicum Examining Committee certifies that the thesis/practicum (and oral examination if required) is:

Approved

(Approved or Not Approved)

☑ Thesis ☐ Practicum

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Regulation of Cholesterol Biosynthesis in Hepatocytes

By

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A thesis submitted to the Faculty of Graduate Studies

in fulfillment of the requirements for the degree of

Master of Science

Department of Physiology

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Abstract

Hypercholesterolemia, a condition of high cholesterol levels in the circulation, poses a major risk for developing cardiovascular disease, such as atherosclerosis. A common method of reducing plasma cholesterol levels relies on the administration of drugs that limit cholesterol synthesis or uptake, many of which have undesirable side effects. Thus, some patients are turning to an alternative treatment, namely natural health products. Natural health products are often equally or even more effective at treating illness than synthetic drugs and may produce fewer side effects. The goal of this study was to identify a natural health product that regulates hepatic cholesterol synthesis by inhibiting HMG-CoA reductase, the enzyme which catalyzes the rate-limiting step of the cholesterol synthesis pathway. Several natural compounds were screened using the human hepatoma cell line HepG2. One compound, berberine, showed great potential as a regulator of cholesterol synthesis and so became the subject of this investigation. Berberine inhibited HMG-CoA reductase activity and decreased cellular accumulation of cholesterol. Berberine was shown to regulate HMG-CoA reductase through activation of metabolic regulator AMP-activated protein kinase, which modifies HMG-CoA reductase post-translationally and thereby decreases its activity. In conclusion, this study demonstrates that the natural health product berberine decreases cholesterol synthesis by activating a cellular signalling pathway to bring about post-translational modification of HMG-CoA reductase, and in doing so, inhibits this enzyme. This novel mechanism supports berberine's potential for a cholesterol-lowering therapy and its role in reducing the risk for cardiovascular disease.

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Abbreviations

ACC	Acetyl-CoA carboxylase
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
BAS	Bile acid sequestrants
BBR	Berberine
bHLH-Zip	Basic helix-loop-helix leucine zipper
CaMK	Calcium/calmodulin-dependent protein kinase
cAMP	Cyclic AMP
CHD	Coronary heart disease
CM	Chylomicron
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FH	Familial hypercholesterolemia
HDL	High density lipoprotein
HMG-CoA	3-hydroxy-3-methylglutaryl Co-enzyme A
IDL	Intermediate density lipoprotein
Insig	Insulin induced gene
LDL-C	Low density lipoprotein cholesterol

LDLR	Low density lipoprotein receptor
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
MRSE	Muscle-related side effects
NCEP ATP	National Cholesterol Education Program Adult Treatment Panel
NHP	Natural health product
NO	Nitric oxide
nSREBP	Nuclear sterol regulatory element binding protein
oxLDL	Oxidized LDL
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SCAP	SREBP cleavage activating protein
SREBP	Sterol regulatory element binding protein
SSD	Sterol-sensing domain
TG	Triglycerides
VLDL	Very low density lipoprotein

Chapter 1

Introduction

1.1 Atherosclerosis and Hypercholesterolemia

1.1.1 Molecular Mechanisms of Atherosclerosis

Atherosclerosis is a type of cardiovascular disease (CVD) with a complex, multi-factorial etiology. In the early stages, the disease is characterized by vascular inflammation, endothelial dysfunction and lipid accumulation in the arterial wall. In the later phases of atherosclerosis, complex vascular lesions or "plaques" form in the vascular wall, which bulge into the vessel lumen, causing narrowing and stiffening of the vessel. Lesion rupture releases components of the core directly into the lumen of the vessel. Rapid coagulation of these factors results in the formation of a blood clot (known as thrombosis), which may block the flow of blood through the vessel, depriving cells and organs of essential nutrients (Halvorsen et al., 2008).

In the early stages of atherosclerosis, an insult to the vascular endothelium triggers an inflammatory response. The insult may be due to shear stress, hypertension, infectious microorganisms, free radicals, elevated and modified lipoproteins, toxins from smoking, or a combination of these and other risk factors. The inflammatory response that results from the injury leads to endothelial dysfunction that alters the normal homeostatic properties of the endothelium (Ross, 1999). The endothelium becomes more adhesive and permeable to inflammatory cells such as leukocytes and platelets, and begins to form vasoactive molecules, cytokines and growth factors. These signalling molecules steadily recruit monocytes to the site of injury, which infiltrate the sub-endothelial space and differentiate into macrophages (Ross, 1999).

Cholesterol is very hydrophobic and for this reason it is transported in the circulation as part of a protein/lipid complex, which increases its solubility. The molecules that carry

cholesterol and other lipids, such as triglycerides and phospholipids, are called lipoproteins. Lipoproteins are categorized into five classes based on their densities. A more detailed description of lipoproteins, their components and their metabolism is found in section 1.2 of this chapter. Cholesterol contained in low density lipoprotein (LDL), the most cholesterol-rich lipoprotein, is a major cause of injury to the endothelium and underlying smooth muscle layer. Figure 1.1 illustrates the role of cholesterol in the development of atherosclerosis.

Due to elevated permeability of the endothelium, LDL can become trapped in the vascular wall, where it undergoes oxidative modification by free radicals released from damaged endothelial cells (Morel et al., 1983). Oxidative modification refers to the removal of electrons from or addition of oxygen to the molecule in question, and may be enzymatic (involving oxidases or peroxidases) or non-enzymatic in nature (Proctor and Reynolds, 1984). Non-enzymatic oxidation reactions involve activated chemical compounds known as "reactive oxygen species" (ROS), which have one or more unpaired electron(s), making them highly unstable and very reactive. Examples include hydroxyl radicals, lipid peroxyls, singlet oxygen, superoxide anion and peroxynitrite (Proctor and Reynolds, 1984). Oxidized LDL (oxLDL) displays "molecular heterogeneity", meaning that LDL can be modified in many different ways and to varying degrees, and the resulting population of oxLDL are not identical to each other. Oxidation of LDL can occur through enzymatic oxidation or through interactions with ROS, and may involve modifications to both protein and lipid components (Steinberg, 1997b). For example, the attack of a free radical on the double bond of a fatty acid results in a lipid peroxidation chain reaction (Young and McEneny, 2001), extensively

modifying lipid components of the LDL molecule. Additionally, cross-linking can occur between protein and lipid components. These and other modifications render the LDL molecule more negatively charged, thereby increasing its affinity for scavenger receptors on macrophages (Steinberg, 1997b). This increased affinity for macrophage receptors is the primary reason oxLDL is believed to be atherogenic (Chisolm and Steinberg, 2000). Macrophages in the sub-endothelial space rapidly internalize oxLDL, become engorged, and thus are classified as foam cells. Foam cells form a fatty streak, the earliest visible form of the mature atherosclerotic lesion. As the disease progresses, cholesterol and foam cells continue to accumulate, and prolonged oxidative stress and inflammation contribute to endothelial dysfunction.

Normal physiological endothelial function depends on a delicate balance between pro-oxidative and anti-oxidant mechanisms in the vascular wall. In the healthy state, these mechanisms favour the production of nitric oxide (NO), a critically important signalling molecule in many metabolic pathways in endothelial function and atherosclerosis. An imbalance in production or bioavailability of NO or its downstream signalling molecules in the vasculature is implicated in the pathogenesis of endothelial and vascular smooth muscle dysfunction (Giles, 2006).

The cytokine-rich environment in the sub-endothelial space stimulates vascular smooth muscle cell migration to a position over the lipid-rich core of the lesion and the muscle cells divide continuously, causing the lesion to bulge into the lumen of the vessel (Ross and Glomset, 1973). The progressively enlarging lesion interferes with nutrient exchange in the cells that make up the vessel wall around the plaque, leading to degeneration of the vessel wall. The damaged area is infiltrated by fibroblasts, which form a cap of collagen-

rich connective tissue over the plaque, thereby stabilizing it. Calcium deposition occurs throughout lesion formation, contributing to stenosis of the vessel. These end-stage lesions are influenced by inflammatory cytokines and are constantly remodelled. Atherosclerotic lesion rupture is associated with a combination of biomechanical forces that are dependent on the thickness of the fibrous cap, the thickness of the lipid core region and positive remodelling of the plaque (Badimon et al., 2002; Bui et al., 2009). The clinical manifestations of atherosclerosis can vary, depending on the site where the plaques develop. Myocardial infarction (commonly referred to as a "heart attack") can occur if plaques form in the coronary arteries and reduce or partly occlude the blood flow to the heart muscle, which requires a steady supply of nutrients to maintain its critical role in blood distribution. Alternatively, a stroke may occur if a plaque ruptures and a blood clot blocks a narrowed, hardened vessel supplying the brain, depriving the brain of oxygen, glucose and other essential nutrients. Other manifestations of atherosclerosis include arterial occlusive disease (peripheral arteries), kidney failure (renal arteries) and aortic aneurisms (aorta) (Saini et al., 2005). In many cases, there are no detectable clinical symptoms of vascular disease before the heart attack or stroke, but by this point, dietary or lifestyle interventions alone are rarely sufficient to improve the condition.

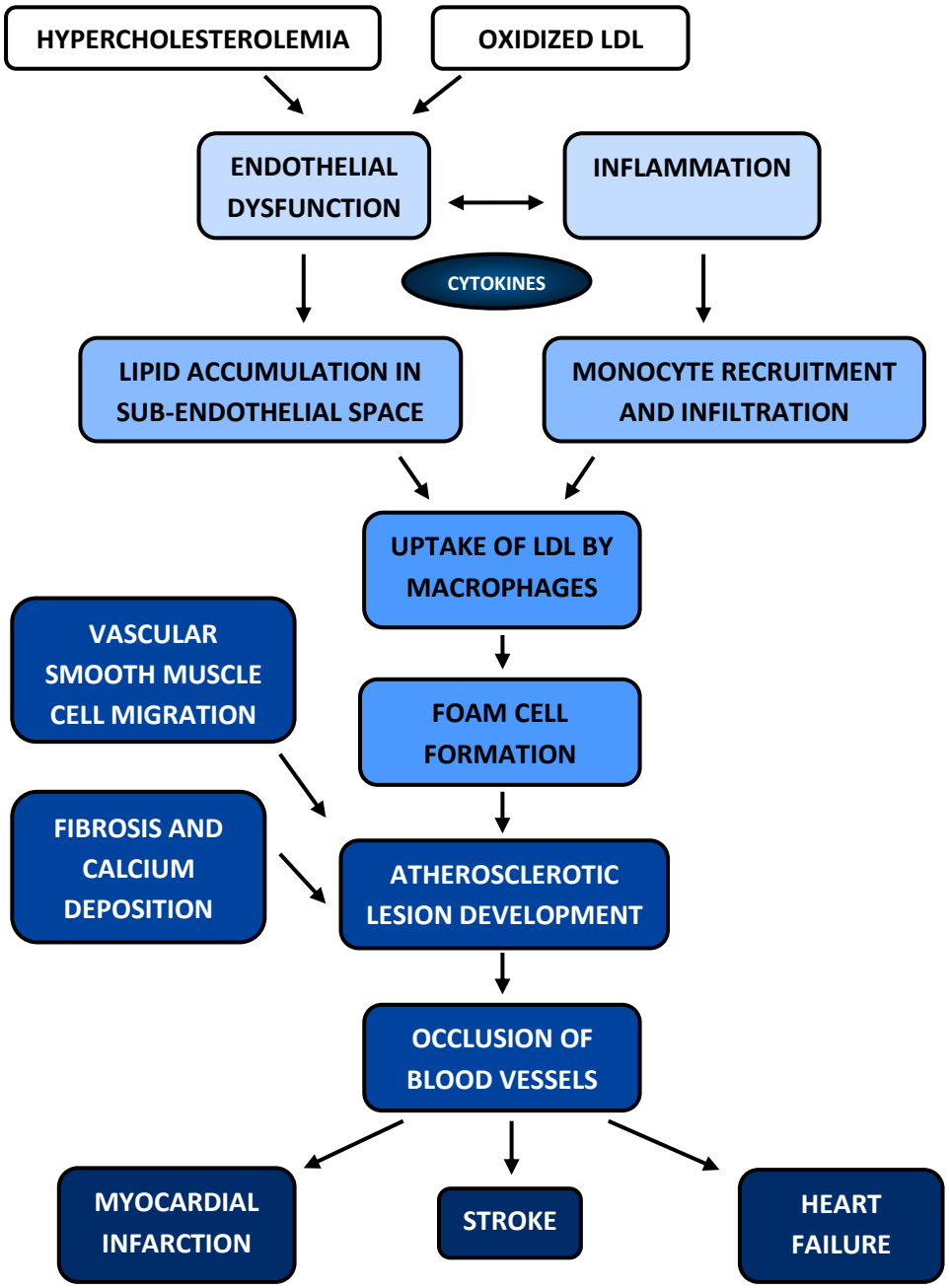

Figure 1.1 The Role of Cholesterol in the Development of the Atherosclerotic Lesion

Cholesterol contributes to the development of atherosclerosis through its role in oxidative stress. In the circulation cholesterol is carried by LDL, and these molecules are highly susceptible to oxidation. The free radicals released upon LDL oxidation damage the endothelium and promote the release of inflammatory cytokines. Oxidized LDL also contributes to foam cell formation. Atherosclerotic lesions develop, and eventually lead to the occlusion of blood vessels. The clinical symptoms associated with the disease vary, depending on which vessels and organs are affected by the blockage of blood flow.

1.1.2 Prevalence and Impact of Atherosclerosis

Cardiovascular diseases (CVD) are the leading cause of death in the Western Hemisphere with atherosclerosis among the most serious of these. Coronary heart disease (CHD), a type of atherosclerosis that results in the occlusion of the coronary arteries of the heart muscle, is the number one killer in the United States (American Heart Association, 2010). In 2005, CVD caused 35% of all deaths in the United States and the healthcare costs for cardiovascular disease in that country were estimated at $475 billion for 2009 (National Institutes of Health, 2008).

The Executive Summary of the Third Report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults – Treatment Panel III (NCEP ATP) focuses on cholesterol as a major risk factor in the pathophysiology of coronary heart disease (The National Cholesterol Education Program, 2001). This assertion is based on the results of numerous animal studies and large-scale clinical trials which have demonstrated that lowering cholesterol will reduce CHD and CVD morbidity and mortality (Scandinavian Simvastatin Survival Study, 1994; Shepherd et al., 1995; Sacks et al., 1996; Downs et al., 1998; LIPID Study, 1998). Although low levels of high density lipoprotein cholesterol (HDL-C) and elevated triacylglyceride (TG) levels have also become essential for risk assessment, plasma LDL-C is still the main target of lipid-lowering therapy (The National Cholesterol Education Program, 2001). It is therefore of utmost importance to develop methods to lower cholesterol, reinstate lipid homeostasis and improve vascular health in dyslipidemic patients.

1.1.3 Risk factors for Cardiovascular Disease

Atherosclerosis is a disease of multi-factorial etiology. The list of risk factors for atherosclerosis continues to grow as the field of cardiovascular disease research expands. The major independent risk factors, of which many are additive, are summarized in Table 1.1. For example, the clustering of abdominal obesity, dyslipidemia (a condition of high LDL-C, high TG and low HDL-C levels), increased blood pressure (hypertension) and insulin resistance is referred to as metabolic syndrome. However, other lipid-related factors are also important in the development of atherosclerosis. Hypercholesterolemia and hypertriglyceridemia often occur simultaneously, but act via distinct pathways to bring about damaging effects on the vasculature. Oxidized LDL is also a major contributor to endothelial dysfunction and inflammation in the early stages of atherosclerosis. Dietary lipids, such as saturated fats and *trans* fats, have received a great deal of attention both in the media and in the scientific community. These fats ultimately influence plasma lipoprotein balance and have consistently been shown to be harmful. In the following section, the mechanisms by which these lipid-based risk factors contribute to the pathogenesis of cardiovascular disease are discussed in more detail. The remainder of this thesis focuses specifically on cholesterol as a risk factor for atherosclerosis and the mechanisms by which cholesterol metabolism is regulated in health and disease states.

Table 1.1 Independent Risk Factors for Atherosclerosis

Non-modifiable risk factors	Behavioural/Metabolic-related risk factors
Age	Cigarette Smoking
Gender	Diabetes Mellitus
Family History	Obesity
	Dyslipidemia
	Blood Pressure (Hypertension)
	Hyperhomocysteinemia

(Pasternak, 2003; Genest et al., 2009)

Age, gender and family history of cardiovascular disease are all non-modifiable risk factors for atherosclerosis. Other behavioural factors, such as cigarette smoking, and metabolic imbalances like hypertension, obesity and dyslipidemia can be curbed or regulated.

1.1.3.1 Hypercholesterolemia and Hypertriglyceridemia

The terms hypercholesterolemia and hypertriglyceridemia refer to conditions of elevated levels of cholesterol and triglyceride in the circulation, respectively. Ideally, levels of plasma LDL-C in humans should be lower than 100 mg/dL, and triglycerides (TG) should be lower than 150 mg/dL (American Heart Association, 2009). Increases in plasma lipid levels correspond with increased risk for developing CVD. Epidemiological studies have consistently demonstrated that LDL-C levels are correlated with CVD risk. The data presented by Grundy and colleagues suggests that for every 1 mg/dL increase in LDL-C, the relative risk for coronary heart disease (CHD) increases by 1% (Grundy et al., 2004). Elevated LDL-C plays a key role in atherosclerotic plaque development and in progressive growth and rupture of the plaque, which causes most of the symptoms of acute CHD (Kruth, 2001). Evidence from controlled clinical trials for lowering LDL-C has corroborated a causal role for LDL-C in atherogenesis and CVD (The National Cholesterol Education Program, 2002). All reports released by the NCEP ATP identify LDL-C lowering as the primary goal of cardiovascular event risk reduction. The guidelines set forth by the NCEP ATP delineate categories of risk for cardiovascular events based on blood cholesterol levels, as shown in Table 1.2 (Grundy et al., 2004; American Heart Association, 2009). However, there is growing support for the theory that the absolute value of LDL-C after reduction is less important than the proportion of the reduction from initial levels (MRC/BHF Heart Protection Study, 2002b), particularly in populations of high and very high risk patients with high LDL-C values (Grundy et al., 2004). In general, the goal of hypercholesterolemia treatment should be a reduction of LDL-C of at least 30% below the baseline (MRC/BHF Heart Protection Study, 2002b).

Elevated TG levels are believed to increase CVD risk through the atherogenic effects of TG-rich remnant particles, which are the products of lipoprotein metabolism in the vasculature. The large lipid and protein aggregates formed in the intestine from dietary lipids are known as chylomicrons. Chylomicrons are the least dense class of lipoproteins (further details provided in section 1.2 of this chapter) The TG core of chylomicrons is removed by lipoprotein lipase, and what remains are lipoprotein remnant particles, which are perhaps sufficiently small to infiltrate the arterial wall and, at elevated levels, contribute to lesion formation (Gianturco et al., 1998; Brewer, 1999). Emerging evidence also demonstrates a critical role for HDL-C in lowering CVD risk; a meta-analysis of 23 trials estimated that a 40% reduction in LDL-C levels combined with a 30% elevation in HDL-C levels would result in a 70% reduction in CVD risk (Brown et al., 2006). The cholesterol contained in HDL is known as "good cholesterol" because of HDL's role in shuttling excess cholesterol back to the liver (a process called reverse cholesterol transport). Further details on cholesterol transport and metabolism are discussed in section 1.2.2.

The underlying mechanisms by which excess cholesterol and TG exert their pathogenic effects on the vasculature are not well defined. However, the sheer volume of fatty molecules in the circulation increases the chance of sub-vascular lipid accumulation and contributes to the production of volatile and damaging lipid peroxides. In addition, it is generally accepted that LDL-C becomes particularly atherogenic when oxidized, as discussed below.

1.1.3.2 Oxidized Lipids

In 1979, the idea of oxidized LDL (oxLDL) contributing to the development of atherosclerosis was set forward by Goldstein and colleagues (Goldstein et al., 1979), when they showed that native LDL underwent a modification that allowed it to be taken up by scavenger receptors on macrophages, which subsequently incorporated the LDL-C into the atherosclerotic plaque. Ten years later, Steinberg et al. (Steinberg et al., 1989) proposed the oxidative modification hypothesis, which was based on the concept that oxidation represents a biological modification of LDL ultimately giving rise to a foam cell. According to the oxidation modification hypothesis, oxLDL contributes to atherogenesis by 1) assisting in monocyte recruitment to the sub-endothelial space by promoting expression of inflammatory cytokines and chemotactic factors, 2) preventing resident macrophages from leaving the sub-endothelial space, 3) enhancing the rate of lipoprotein uptake leading to foam cell formation, and 4) leading to endothelial cell injury and endothelial dysfunction through its cytotoxic effects (Quinn et al., 1985). Many studies support this hypothesis (Steinberg, 1997a; Chisolm and Steinberg, 2000; Glass and Witztum, 2001; Steinberg and Witztum, 2002; Stocker and Keaney, 2004). The role of oxidative stress in CVD has been extensively studied in recent years and several additional mechanisms by which oxLDL contributes to the pathogenicity of atherosclerosis have been demonstrated since the original hypothesis was put forward. For example, it has been shown that oxLDL decreases plaque stability by activating subsets of smooth muscle cells and macrophages to produce gelatinase, an extra-cellular matrix enzyme that is involved in remodelling (Holvoet, 2008). OxLDL also plays a key role in activating many signalling pathways which mediate their effects via

transcriptional factors and up-regulate genes involved in the inflammatory response, oxidative stress processes or cell cycle regulation (Maziere and Maziere, 2009). Thus, it is clear that oxidized lipids pose a certifiable risk to vascular health.

Accordingly, a large body of research has been devoted to preventing oxidation or counteracting its effects. Optimal vascular function is dependent on the balance between oxidant and anti-oxidant mechanisms, which determine the integrity and responsiveness of the endothelium. It was anticipated that dietary anti-oxidants would protect the endothelium and promote vascular health. While it has been observed that people who consume diets rich in fruits and vegetables (many of which are rich in anti-oxidants) have lower risks of cancer, cardiovascular disease and all-cause mortality (Doll and Peto, 1981), the usefulness of anti-oxidants and anti-oxidant vitamins in counteracting oxidative stress and in reducing the harmful manifestations of cardiovascular disease is controversial. In light of the oxidative modification hypothesis, which states that oxLDL is a key factor in atherogenesis as it contributes to foam cell and fatty streak formation, researchers and clinicians have reasoned that these pathological effects might be impeded by treatment with anti-oxidants. To that effect, many animal studies and several large-scale trials have been conducted, with mixed results. While studies in cells and animals have yielded promising outcomes (Beetens et al., 1986; Kita et al., 1987; Aviram et al., 1998), prospective randomized clinical trials have not provided the answers hoped for, in most cases having no effect on human CVD risk.

The major anti-oxidant vitamins present in the body are vitamin E, β-carotene, lycopene and flavanoids. The first two of these are lipid-soluble and are present in the cell membrane and in lipoprotein particles. Vitamin C, on the other hand, is a water-soluble

anti-oxidant vitamin commonly obtained from fruits and vegetables (Steinberg, 1992; Reaven et al., 1993). Anti-oxidant vitamins are believed to exert their effects by neutralizing free radicals and thus protecting cells and lipoproteins from oxidative damage. Some of these (vitamins C and E, β-carotene, lycopene) have been reported to reduce LDL oxidation or LDL uptake by macrophages (Jialal et al., 1991; Reaven et al., 1993). The large-scale trials focused on cardiovascular outcomes, such as primary and secondary prevention of cardiovascular events and disease progression. In the HOPE trial, no significant effect of anti-oxidant vitamins (vitamin E) was demonstrated on cardiovascular disease (Yusuf et al., 2000), and the same result was obtained in the Heart Protection Study, in which the effect of Vitamin E, C and β-carotene on cardiovascular outcomes were tested (MRC/BHF Heart Protection Study, 2002a). In the GISSI-Prevenzione Trial, patients were treated with omega-3 polyunsaturated fatty acids (PUFA) and/or Vitamin E, and only those treated with omega-3 PUFA experienced a protective effect in preventing cardiovascular events (Poschl et al., 1999). The role of oxidation of lipids and/or LDL molecules in human atherosclerosis development is still somewhat controversial in light of these results.

Table 1.2 Risk Categories and Target LDL-C Levels in Cardiovascular Disease

Risk Level	Current Plasma LDL-C	Target Plasma LDL-C
Very high risk patients (established vascular disease and multiple risk factors)	190 mg/dL and above	<70 mg/dL or 50% ↓
High risk patients (those with CHD or CHD risk equivalents and a 10-year risk of >20%)	160-189 mg/dL	<70 mg/dL or 50% ↓
Moderate risk patients (those with ≥2 risk factors and 10-year risk of 10-20%)	130-159 mg/dL	<70 mg/dL or 50% ↓
Low risk patients (those with 0-1 CHD risk factors)	Less than 100-129 mg/dL	

(Pasternak, 2003; Genest et al., 2009)

The Canadian Cardiovascular Society released updated guidelines for treatment of dyslipidemia in 2009. The risk categories described above are based on the results of the Framingham Heart Study, a long-term, multi-generational, ongoing clinical trial begun in 1948 which has provided researchers and physicians with much of the epidemiology of hypertensive or arteriosclerotic cardiovascular disease (Kannel et al., 1979). The estimates of risk provided by the Framingham study are generally considered to be very good and the study is widely cited.

1.1.3.3 Saturated Fats and *Trans* Fats

All fats and oils are composed of triglycerides (TG), which are made up of a glycerol molecule with three fatty acid molecules attached (Figure 1.2). Saturated fatty acids are solid at room temperature (like butter), whereas unsaturated fatty acids are liquid at room temperature (oil). Unsaturated fatty acids have at least one double bond between carbon atoms and can take either the *cis* (Latin for "on the same side") or *trans* (Latin for "on the other side") configurations. Figure 1.3 illustrates the structural differences in oleic acid when a *cis* double bond replaces a *trans* double bond.

Saturated fatty acids (SFA) are found naturally in dairy products such as cream and cheese, animal fats such as lard and tallow, and in coconut oil and palm kernel oil. Diets high in SFA have been correlated with atherosclerosis and CHD. It is well established that SFA increase plasma LDL-C and TG levels (Grundy and Denke, 1990; Mensink, 1993; Mensink et al., 2003; Rivellese et al., 2003). In 1957, the American Heart Association recommended that polyunsaturated fatty acids (PUFA) replace SFA in the diet (Page et al., 1957). Since then, many Westerners and Western food manufacturers have made an effort to reduce their SFA intake and the SFA present in food products. A report from the NCEP ATP recommends that SFA make up less than 7% of calories for optimal LDL-C lowering (The National Cholesterol Education Program, 2001). However, it appears that much of the SFA previously consumed have now been substituted by *trans* fatty acids, which may be even more harmful to vascular health and lipid homeostasis than SFA.

Trans fatty acids (TFA) are long chain unsaturated fatty acids which have one or more double bonds in a *trans* configuration. It is thought that the flat, linear structure of TFA

allows these molecules to stack together more closely than if they were in the bulkier *cis* configuration. TFA are not typically found in significant amounts in nature, but do occur naturally in trace quantities in dairy products and meat. However, the majority of TFA consumption in the Western diet comes from processed foods, such as crackers, cookies and donuts. TFA are created artificially by partial hydrogenation of saturated fats, a process which decreases the requirement for refrigeration and increases shelf life of foods that contain TFA (Teegala et al., 2009). It has now been demonstrated that TFA have detrimental effects on cardiovascular health. In fact, TFA have recently been shown to be unequivocally atherogenic (Bassett et al., 2009). TFA have been shown to increase LDL-C and TG, while decreasing HDL-C, thus promoting dyslipidemia. They also increase levels of circulating markers of systemic inflammation and endothelial dysfunction (Mozaffarian et al., 2004; Lopez-Garcia et al., 2005), cause calcification of arterial cells (Kummerow et al., 1999; Sun et al., 2007b), and inhibit cyclooxygenase, an enzyme which is necessary for the regulation of blood flow (Kummerow et al., 2007). Recent research suggests TFA may also play a role in the development of other chronic diseases such as adiposity and type II diabetes (Micha and Mozaffarian, 2009). Epidemiological data suggest that when *trans* fat percentages go up, death rates rise; when *trans* fat goes down, death rates decrease (National Center for Health Statistics and U.S. Department of Health and Human Services, 2007). Based on the premise that TFA are not essential and provide no known benefit to human health, and that TFA not only increase LDL-C but also decrease atheroprotective HDL (Judd et al., 1994; Mensink et al., 2003), the National Academy of Sciences recommends that the consumption of TFA be eliminated or be less than 1% of total energy intake (Food and Nutrition Board, 2005).

In North America and Europe, recent media attention and policy initiatives have led to widespread product reformulations in a effort to reduce TFA in many food products (Ratnayake et al., 2009).

In summary, many types of lipids contribute to atherosclerosis. Dietary intake of saturated fats, *trans* fatty acids and foods rich in cholesterol and triglycerides can affect the lipoprotein balance. High levels of LDL-C have adverse effects on vascular health. Oxidative stress plays an important role in endothelial dysfunction and vascular lipid accumulation. Mechanistic studies and large-scale clinical trials have demonstrated the harmful effects of these fats, and have led to recommendations for lifestyle and dietary changes and the development of pharmacological therapies. In the following section, pharmacological treatments for hypercholesterolemia are discussed.

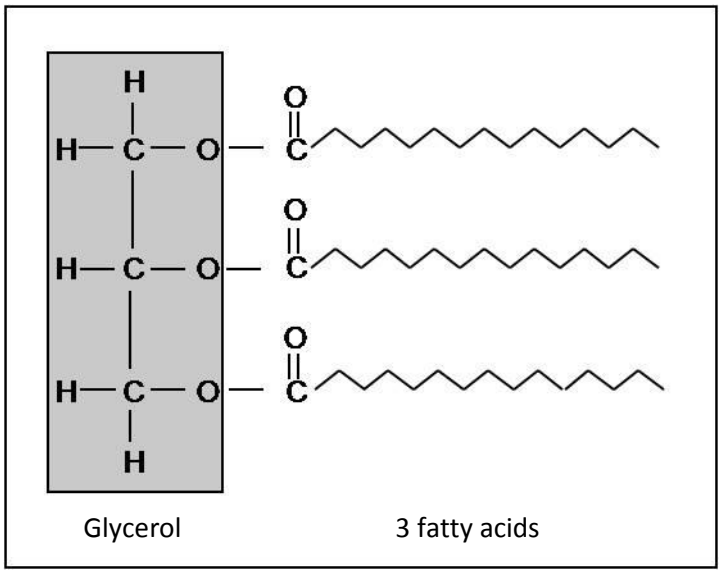

Figure 1.2 Structure of a Triglyceride

Triglycerides are made up of a glycerol "backbone" with three fatty acid "tails".

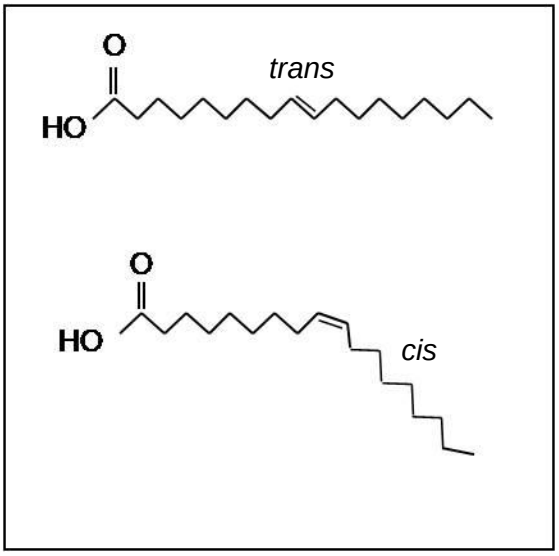

Figure 1.3 Fatty acid (oleic acid) with *cis* and *trans* double bond conformations.

Oleic acid serves as an example to demonstrate the different conformations resulting from *cis* and *trans* double bonds. It is thought that the *trans* configuration allows for better stacking properties of these fats, and may contribute to their accumulation in atherosclerotic lesions. The "kink" caused by the *cis* double bond alters the properties of the fatty acid, making it less "stackable" and, therefore, less atherogenic.

1.1.4 Current Drug Treatments for Hypercholesterolemia

Aside from dietary interventions, such as avoiding saturated fats and *trans* fats, there are also several pharmacological treatments available to reduce plasma cholesterol levels and improve the lipid profile in humans. The most commonly prescribed drugs for cholesterol-lowering are the "Statins", inhibitors of HMG-CoA reductase. Fibrates, another class of drug, interfere with triglyceride metabolism and also have a LDL-lowering effect. Lastly, drugs which act in the intestine to block cholesterol absorption or bile acid reabsorption are useful in reducing plasma cholesterol, as they deplete the hepatic cholesterol pool. These three classes of drugs are described in more detail below.

1.1.4.1 Cholesterol Synthesis Inhibitors (Statins)

Statins are a class of drugs that inhibit the enzyme HMG-CoA reductase, which is responsible for catalyzing the rate-limiting step in the *de novo* synthesis of cholesterol. Six statins are currently on the market: lovastatin and pravastatin are natural products of fungal origin, simvastatin is a semi-synthetic derivative, and fluvastatin, rosuvastatin and atorvastatin are synthetic HMG-CoA reductase inhibitors. Among these, atorvastatin (Lipitor) was found to be the most effective drug in reducing LDL-C levels (Jones et al., 1998). All statins have structurally similar components to HMG-CoA, the substrate for HMG-CoA reductase (Figure 1.4), and act by binding the enzyme's catalytic site, thus preventing access of the substrate to the binding site (Istvan and Deisenhofer, 2001). Subsequently, there is a reduction in cholesterol synthesis and the intracellular cholesterol levels drop. This decline is sensed by a negative feedback mechanism, and consequently genes regulating lipid synthesis and uptake are activated. The primary effect of statins is hepatic cholesterol biosynthesis inhibition, resulting in improved LDL-C clearance from

the plasma through LDL receptors (LDLR) in the liver. Statins also reduce the release of lipoproteins from the liver into the circulation (Arad et al., 1990; Arad et al., 1992). In this way, statins reduce plasma LDL-C and lower the risk of CVD.

Several large scale trials have established the benefits of statin treatment in primary and secondary CVD prevention (Scandinavian Simvastatin Survival Study, 1994; Shepherd et al., 1995; Sacks et al., 1996; Downs et al., 1998; LIPID Study, 1998; Sacks et al., 1998; MRC/BHF Heart Protection Study, 2002a; Sever et al., 2003). Statins have been demonstrated to convey their effects primarily through LDL-C lowering (decrease of 20-60%), but also through a reduction in TG (decrease of 5-35%), depending on the drug, dosage and the patient's baseline levels, leading to an average risk reduction of approximately 25-30% (Davidson et al., 1997; Hou and Goldberg, 2009). A minor increase in HDL is observed (maximally 10-15%) (Jones et al., 1998; Jones et al., 2003) and a halt in progression or even regression of coronary lesions occurs, as measured by angiography (Brown et al., 1990; Blankenhorn et al., 1993). There is also emerging evidence that statins have pleiotropic effects beyond those related to lipid-lowering in CVD patients. It has been suggested that these effects might be mediated through a reduction in circulating hepatic cholesterol precursors. One group of precursors, the isoprenoids, are required for post-translational modification of signalling molecules involved in atherogenic cell proliferation and damaging oxidative processes (Liao, 2002). Upon examination of human plaques *ex vivo*, it was suggested that a shortage of isoprenoids contributes to atherosclerotic plaque stability through an increase in collagen content and a decrease in the activity of metalloproteinases (enzymes involved in extra-cellular matrix remodelling). Statins have also been shown to counteract inflammatory

processes, which provide benefits to patients with chronic inflammatory disease regardless of lipid levels (Nissen et al., 2005; Ridker et al., 2005), and to improve endothelial function and blood flow regulation through their actions as antioxidants (Carneado et al., 2002; Mitani et al., 2003).

Statins are highly effective and relatively safe drugs for many patients, but it becomes increasingly clear that a subset of the population cannot tolerate statins, or cannot tolerate them at a dose high enough to reach their target lipid levels. A range of studies support a dose relationship for many statin adverse effects (Bays, 2006; Cziraky et al., 2006; Silva et al., 2006; Afilalo et al., 2007; Silva et al., 2007). The best recognized and most commonly reported adverse effects are muscle pain (myositis), fatigue and weakness (Scott et al., 1991; Wierzbicki et al., 1999), which in rare cases results in severe or fatal rhabdomyolysis (the breakdown of muscle fibres), leading to acute renal failure (Paoletti et al., 2002; Bruckert et al., 2005; Mukhtar and Reckless, 2005; Baer and Wortmann, 2007). There have also been reports of elevation of liver transaminases, which is a marker for disruption of healthy liver function (Cannon et al., 2004; LaRosa et al., 2005). Other adverse effects can occur due to interactions between statins and other drugs. For example, the risk for rhabdomyolysis increases when statins and fibrates are taken in combination. Statins are metabolized by the cytochrome P450 enzymes CYP3A4 and CYP3A5. The former is inhibited by compounds called furanocoumarins found in grapefruit juice (Willrich et al., 2009). The inhibition of CYP3A4 increases the levels of the statin in the body and may contribute to the risk of dose-related effects.

Other than muscle-related side effects, these negative effects of statins are relatively uncommon or can be avoided by simply eliminating the consumption of

furanocoumarins. However, many patients who do experience muscle pain and cannot continue statin therapy are failing to reach their lipid-lowering targets and remain at risk for cardiovascular events. Consequently, alternative treatments for lipid lowering are highly sought after, to be employed as a replacement for statin therapy or in combination with statins so that the dosage can be reduced.

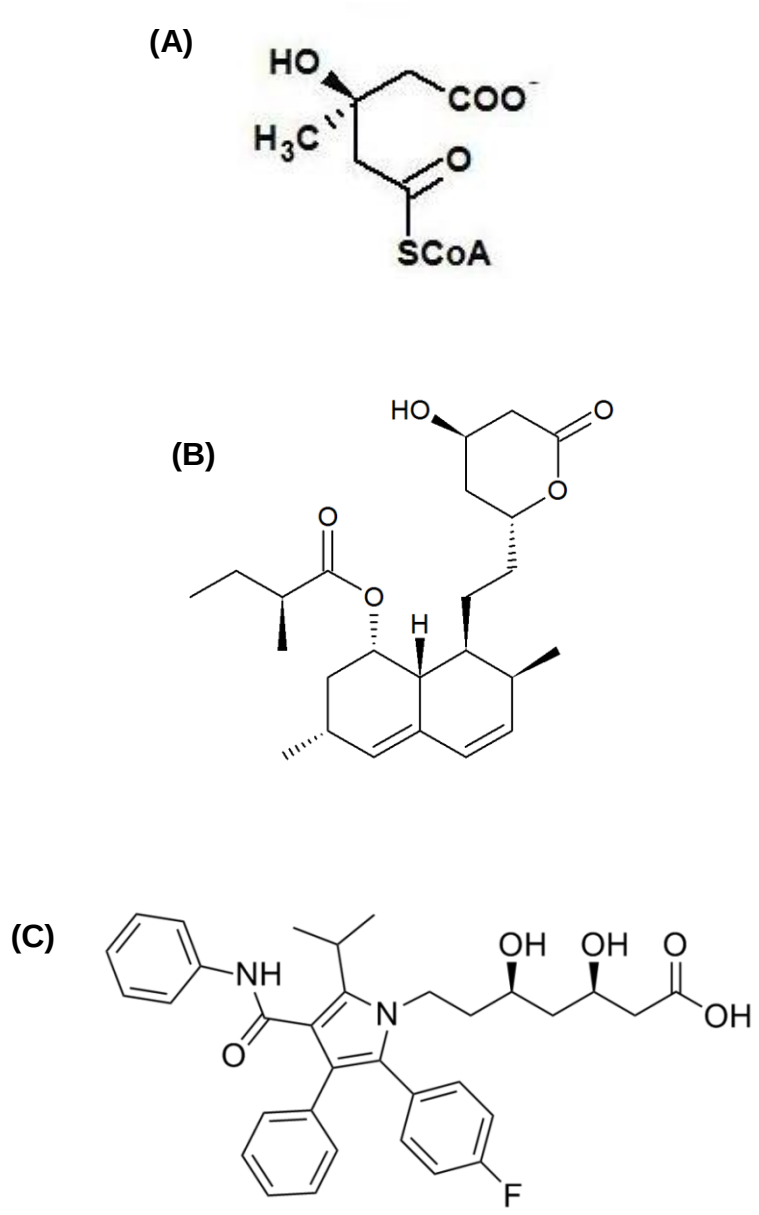

Figure 1.4 The Structure of HMG-CoA and Structural Analogues Lovastatin and Atorvastatin.

HMG-CoA (A) is the substrate of HMG-CoA reductase. The HMG-CoA inhibitors lovastatin (B) and atorvastatin (C) are structural analogues of HMG-CoA.

These images are in the public domain.

1.1.4.2 Fibrates

Fibrates are drugs that interfere with triglyceride metabolism and synthesis, which occurs primarily in the liver, but also affect LDL-C levels. Several fibrates commonly prescribed include gemfibrozil, fenofibrate, clofibrate, ciprofibrate and bezafibrate. The action of fibrates is mediated through peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear receptor superfamily of ligand-induced transcription factors. The binding of fibrates to PPARα causes it to form a heterodimer with another nuclear receptor, retinoid X receptor. The complex then binds to peroxisome proliferator response elements, thereby modulating expression of genes that regulate lipid and fatty acid metabolism and inflammation (Rosenson, 2008). Specifically, activation of PPARα results in increased β-oxidation of fatty acids in the liver, decreased hepatic triglyceride secretion, and increased lipoprotein lipase activity. This leads to improved lipolysis and VLDL clearance, and improved clearance of lipoprotein remnant particles (Barter and Rye, 2006). Fibrates are normally used in combination therapy with statins (Steiner, 2007), since they are somewhat less effective than statins at lowering LDL-C (10-25% reduction), but also decrease triglycerides by 30-60% and increase HDL (up to 10%), and may also have benefits in treatment of metabolic syndrome (Brown, 1987; Fruchart et al., 2001; Fievet and Staels, 2009).

1.1.4.3 Cholesterol Absorption Inhibitors and Bile Acid Sequestrants

The cholesterol absorption inhibitor ezetimibe is the first drug of its class to be marketed. Ezetimibe acts in the small intestine to selectively inhibit the transport of cholesterol into enterocytes. The precise target of ezetimibe was discovered only recently to be

Niemann-Pick C1-Like 1, a protein located in the brush border of enterocytes (Garcia-Calvo et al., 2005). The effect of ezetimibe treatment is to increase cholesterol excretion, much of which is in the form of bile acids, thus decreasing hepatic cholesterol levels and stimulating LDL-C uptake from the circulation in order to maintain adequate levels to replenish the bile acid pool. Ezetimibe alone decreases circulating LDL-C by 15-25%, but it is often used in combination with statins, in which case its effects are additive (Dujovne et al., 2002).

Bile acid sequestrants (BAS) are another class of drugs that act in the small intestine to interfere with cholesterol absorption. There are three BAS currently in use, cholestyramine, colestipol, and colesevelam. BAS are large polymers that bind negatively charged bile acids and bile salts in the small intestine, which interrupts the enterohepatic circulation of bile acids (Grundy et al., 1971; Shepherd et al., 1980). Bile acids are normally reabsorbed in the large intestine and recycled in the liver. BAS promote the elimination of bile acids and can deplete the endogenous cholesterol pool by about 40%, increasing conversion of cholesterol into bile in the liver through the stimulation of 7α hydroxylase, the first enzyme of bile acid synthesis. The subsequent decline in intracellular cholesterol levels up-regulates expression of LDLR and improves plasma LDL-C clearance (Rudling et al., 1990). Although BAS monotherapy effectively lowers LDL-C, BAS are normally used in combination with statins, since the diversion of cholesterol to bile acids also leads to up-regulation of cholesterol and synthesis and VLDL output into the circulation (Beil et al., 1982). In general, this combination is most effective in patients where high LDL-C is the main lipid abnormality. Combinations of BAS with non-statin lipid-lowering agents, such as fibrates and ezetimibe may be useful

in those patients who require intensive lipid-lowering, but cannot tolerate statins (Insull, 2006).

1.2 Regulation of Cholesterol Metabolism

1.2.1 Lipoproteins

Hepatocytes are the major cell type in the liver, constituting 70 – 80% of the cytoplasmic mass of the liver. They are also the primary cholesterol synthesizing cells in the body. All cells are capable of producing cholesterol *de novo*, since they require the sterol as a membrane component for growth or repair and in specialized cases, such as in the adrenals, skin and gonads, use it as a precursor to hormones or vitamin D. However, hepatocytes are the only cell type that synthesize and package cholesterol for delivery to other tissues. Cholesterol and triglycerides are transported as components of lipoproteins, of which there are several classes, categorized by density. The cell assembles a hydrophobic core of cholesterol esters and TG, and incorporates free cholesterol, phospholipids, and a protein component into the outer layer. Each lipoprotein class has a characteristic protein and lipid profile, details of which are given in Table 1.3. Figure 1.5 illustrates the structure of an LDL molecule.

Table 1.3 Lipoprotein family densities and protein/lipid profiles

Density (g/mL)	Class	Primary Protein Components	Diameter (nm)	% Pro	% Cho	% PL	% TG
>1.063	HDL	apoA-I, apoC, apoD, apoE	5-15	33	30	29	8
1.019-1.063	LDL	apoB-100, apoC, apoE, apo(a)	18-28	25	50	21	4
1.006-1.019	IDL	apoE, apoB-100, apoC	25-50	18	29	22	31
0.95-1.006	VLDL	apoE, apoB-100, apoC	30-80	10	22	18	50
<0.95	CM	apoB-48, apoA, apoC, apoE, apoH	100-1000	<2	8	7	84

(Alaupovic, 1972)

Lipoproteins are categorized into five classes based on their densities. The highest density classes have a greater proportion of protein, while the lower density classes consist primarily of lipids. HDL: high density lipoprotein; LDL: low density lipoprotein; IDL: intermediate density lipoprotein; VLDL: very low density lipoprotein; CM: chylomicron; Pro: protein; Cho: cholesterol; PL: phospholipid; TG: triglyceride

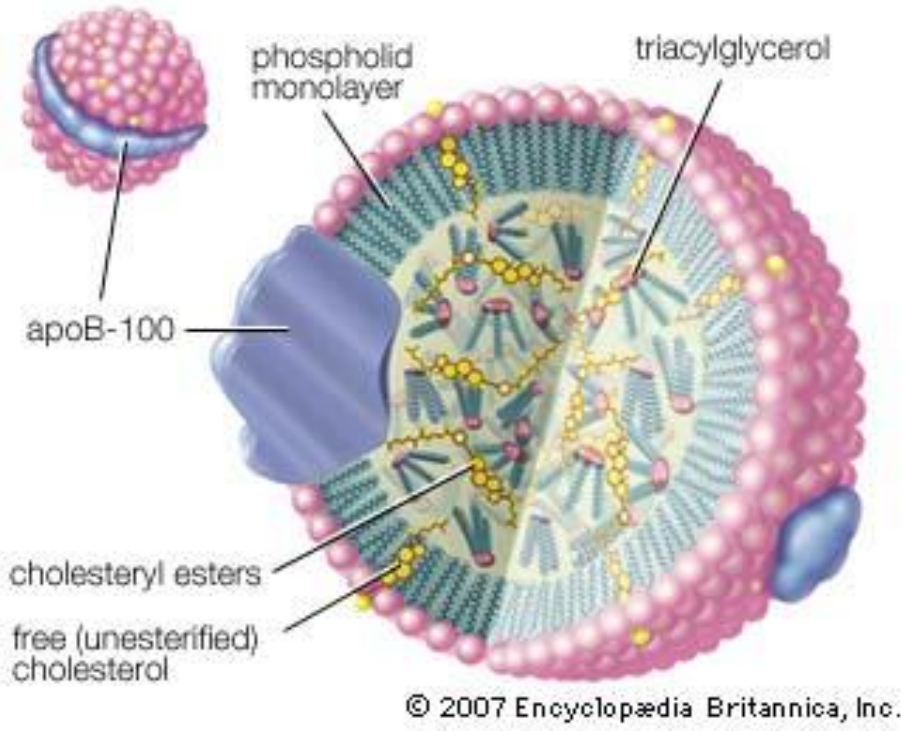

Figure 1.5 Cutaway view of low-density lipoprotein.

Low density lipoprotein contains a hydrophobic core of triglycerides (triacylglycerides) and cholesterol esters. The outer layer is composed of phospholipids, free cholesterol and protein (apoB-100).

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1.2.2 Regulation of Cholesterol Transport

The liver synthesizes very low density lipoprotein (VLDL), a triglyceride (TG)-rich lipoprotein, to transport endogenous triglycerides and cholesterol to other tissues during fasting and the postprandial state. VLDL travels via the bloodstream to peripheral tissues, such as skeletal muscle and adipose tissue, where it unloads TG with the assistance of the endothelial membrane-bound enzyme lipoprotein lipase. Free TG are taken up by the surrounding tissue, broken down into free fatty acids and monoacylglycerides, and used as an energy source (in the case of muscle) or stored for future energy needs (in the case of adipose tissue). As VLDL shrinks, it interacts with HDL to give up extra phospholipids and free cholesterol, which the HDL shuttles back to the liver in a process termed reverse cholesterol transport. The two lipoproteins also exchange protein components. HDL contains the enzyme lecithin:cholesterol acyltransferase, which allows it to esterify free cholesterol as it is received. HDL can obtain excess cholesterol not only from VLDL, but also directly from the cell membranes of peripheral tissues (Barter and Rye, 1996). The majority of free cholesterol in a cell is associated with the membrane, while a small amount is stored in the form of cholesterol esters. As VLDL loses its lipid components to HDL and the surrounding tissues, it is eventually converted via intermediate density lipoprotein (IDL) into LDL, and the cholesterol-rich LDL can be taken up by cells with a requirement for cholesterol by receptor-mediated endocytosis. The remainder of LDL in the bloodstream travels back to the liver. Uptake of LDL occurs through the LDL receptor (LDLR) (Brown et al., 1975; Goldstein and Brown, 1976). LDL binds the LDLR on the cell surface and is rapidly internalized, delivering the LDL load to endosomes and eventually to lysosomes, where

the cholesterol esters contained in the LDL are hydrolyzed to free cholesterol and the receptors are recycled back to the cell surface. The dismantled lipoprotein fulfills a cellular function crucial to the maintenance of cholesterol homeostasis. Firstly, the incoming cholesterol suppresses the activity of HMG-CoA reductase, reducing cholesterol synthesis in the cell (Bilheimer and Levy, 1973; Brown et al., 1974; Bilheimer et al., 1975). Secondly, it activates a microsomal cholesterol esterifying enzyme called acyl-CoA cholesterol acyl transferase, ensuring that excess cholesterol can be re-esterified and stored (Goldstein et al., 1974; Brown et al., 1975). And lastly, the incoming cholesterol suppresses synthesis of LDLR, reducing further uptake of LDL from the circulation and protecting the cell against an over-accumulation of cholesterol (Brown and Goldstein, 1975). Synthesis of LDLR is transcriptionally controlled by mechanisms aligned with those of cholesterol synthesis. When the cell requires more cholesterol, it synthesizes more LDLR and inserts it in the plasma membrane. When cholesterol levels in the cell are sufficient, the cell down-regulates both cholesterol biosynthesis and LDLR synthesis. Details of this regulatory pathway are described in section 1.2.3.

The LDLR uptake pathway is disrupted in individuals suffering from familial hypercholesterolemia (FH), a genetic defect in the LDLR that prevents efficient uptake of LDL from the circulation. Heterozygous FH affects 1 in 500 individuals, placing the disorder among the most common inherited diseases in humans (Brown and Goldstein, 1979). In these individuals, a portion of the LDL receptor is defective or lacking entirely. As a consequence, the negative feedback loop is interrupted and cells continue to produce large amounts of cholesterol (Brown and Goldstein, 1976). The resulting high levels of

plasma cholesterol place these individuals at risk for developing atherosclerosis prematurely, and many would die of hypercholesterolemia-related heart disease if they were not treated to lower their blood cholesterol. In homozygous FH, almost all of the individuals' LDL receptors are affected and receptor activity may be as low as 1% of normal (Hopkins, 2003). The resulting 4- to 5-fold increases in levels of plasma LDL-C lead to the development of advanced cardiovascular disease early in life. Homozygous FH patients often die by the age of 30 from myocardial infarction. Fortunately, homozygous FH is a relatively rare condition, affecting about 1 in 1,000,000 individuals (Durrington, 2003).

De novo synthesis is not the body's only source of cholesterol – it can also be obtained from the diet. The typical human diet contains 200-500 mg cholesterol per day and 30-60% of cholesterol ingested is absorbed (Levy et al., 2007). This equates to about 30% of the body's daily cholesterol needs; the remainder is synthesized endogenously. In the intestine cholesterol and TG are incorporated into chylomicrons (CM), low density globules of dietary lipids and protein components, which enter the bloodstream and travel to various tissues. TG are unloaded from the CM by the action of vascular endothelium-bound lipoprotein lipase and the diminished CM remnants are eventually taken up by the liver and broken down into their lipid and protein components for repackaging. Cholesterol in the liver is an important precursor for the synthesis of bile acids. Bile acids are synthesized in the liver, stored in the gallbladder and added to the contents of the small intestine to aid in digestion. About 2 g of cholesterol per day is secreted into the small intestine in the form of bile acids. Up to 95% of the bile acids are reabsorbed in the distal part of the small intestine and returned to the liver (Wong et al., 1994; Russell,

2003). The amount of cholesterol in the intestine derived from bile acids comprises up to 5-fold the amount taken in from the diet. For this reason, cholesterol must be excreted daily in the feces to avoid excessive accumulation in the body. This pathway is affected by drugs targeting cholesterol absorption and excretion, such as ezetimibe and bile acid sequestrants. Figure 1.6 summarizes the transport and metabolism of cholesterol in the human body.

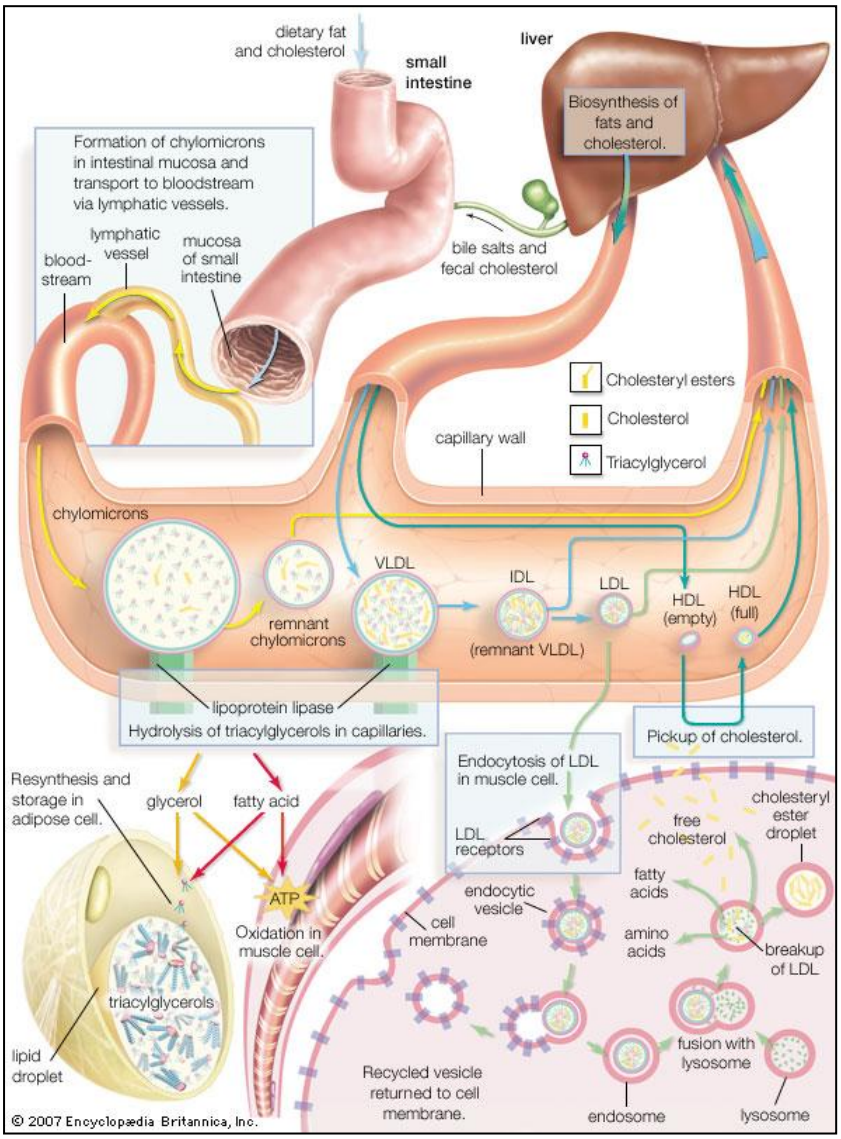

Figure 1.6 Cholesterol transport and metabolism in the human body.

Cholesterol in the body is obtained from the diet or through *de novo* synthesis in the liver. Dietary lipids are absorbed into the circulation and may serve as energy sources in muscle. Cholesterol can be stored in adipose tissue and liver. Hepatic cholesterol is shuttled via lipoproteins to various tissues as required. Hepatic cholesterol is also used in the synthesis of bile acids, which assist in the breakdown of food substances in the small intestine.

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1.2.3 Regulation of Cholesterol Synthesis

In hepatocytes, the primary cholesterol-synthesizing cells of the body, cholesterol homeostasis is maintained via a series of negative feedback loops (Andersen et al., 1982). Simply stated, if the cell has sufficient cholesterol, it down-regulates its synthesis machinery until cholesterol levels drop, at which point the cell reactivates the synthesis pathway to increase levels once again. Cholesterol itself acts as a regulatory molecule at several points in the pathway; this is also true of some other sterol derivatives. The cholesterol synthesis pathway is a series of more than 30 enzymatic reactions, but only a handful of enzymes serve as key regulatory points (Figure 1.7). The enzyme recognized as catalyzing the rate-limiting step is HMG-CoA reductase. The reductase is extensively controlled by transcriptional, translational and post-translational mechanisms, described in detail below. HMG-CoA reductase converts HMG-CoA to mevalonate, which is an important intermediate for the synthesis of signalling molecules such as the isoprenoids, dolichol and ubiquinone. Cells take up LDL-C via the LDLR. Subsequent degradation of the LDL particles releases free cholesterol into the cell, which down-regulates the activity of HMG-CoA reductase, decreases its transcription and increases its rate of degradation (Edwards et al., 1983). Full obstruction of HMG-CoA reductase can only be achieved with both lipoprotein-derived sterols and a non-sterol mevalonate derivative, thus the regulation of this enzyme is termed multivalent negative feedback (Goldstein and Brown, 1990). HMG-CoA reductase is the primary target of statin drugs, which have been shown to be successful in lowering plasma cholesterol in several large scale trials (Scandinavian Simvastatin Survival Study, 1994; Shepherd et al., 1995; Sacks et al., 1996; Downs et al., 1998; LIPID Study, 1998). Inhibition of HMG-CoA reductase up-

regulates LDLR, enhancing plasma cholesterol clearance and lowering LDL-C levels in the circulation. HMG-CoA synthase is the enzyme that condenses acetoacetyl-CoA and acetyl-CoA to HMG-CoA. HMG-CoA synthase is under transcriptional control and it is down-regulated in the presence of sufficient cellular cholesterol (Ayte et al., 1990; Hua et al., 1993). Squalene synthase is likewise down-regulated by high levels of intracellular cholesterol, funnelling excess mevalonic acid into non-sterol synthetic pathways (Tansey and Shechter, 2000). Squalene synthase inhibitors are hypothesized to lower plasma cholesterol by blocking hepatic cholesterol synthesis and causing an up-regulation of LDLR, similar to the action of statins, but further downstream in the cholesterol biosynthesis pathway. Drugs targeting this enzyme for lipid-lowering purposes are under development (Burnett, 2006; Tavridou et al., 2006).

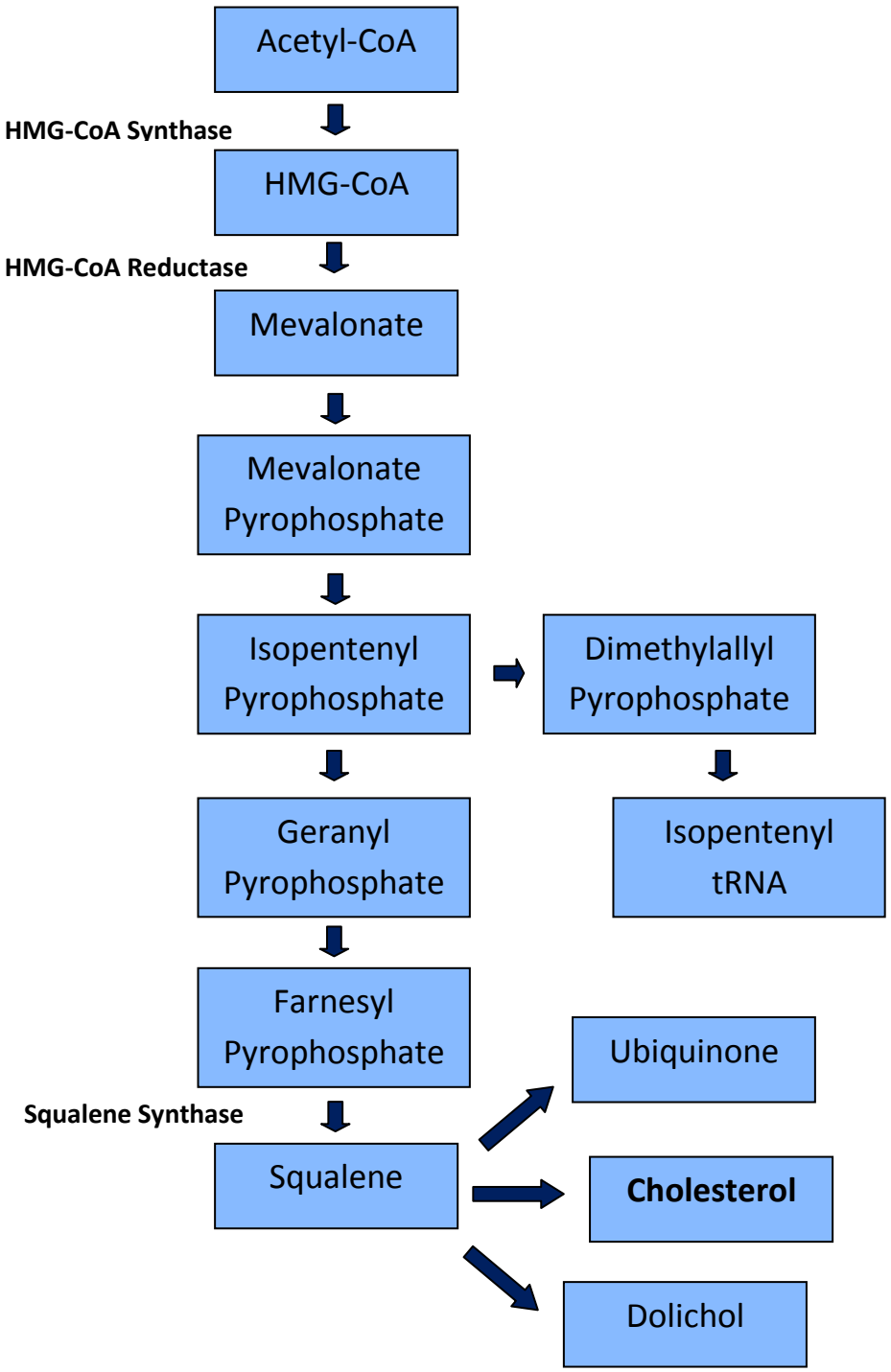

Figure 1.7 The Major Steps of the Cholesterol Biosynthesis Pathway

The cholesterol synthesis pathway involves over 30 enzymatic reactions, starting with the simple two-carbon substrate acetate. Besides cholesterol, several metabolically important molecules are products of this pathway, including ubiquinone, dolichol and the isoprenoids.

1.2.3.1 Transcriptional Regulation of HMG-CoA Reductase

The primary transcription factors responsible for regulating lipid biosynthesis in the liver are the sterol regulatory element binding proteins (SREBP). Three SREBPs are found in mammalian cells, SREBP-2, SREBP-1a and -1c, of which the latter two are isoforms produced from the same gene through use of different promoters and alternative splicing (Brown and Goldstein, 1997). SREBPs belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors, but unlike other bHLH-Zip proteins, they are synthesized as inactive precursors in the endoplasmic reticulum (ER) and inserted into the ER membrane. They are hairpin-shaped with both amino and carboxyl termini facing the cytosol, and under sterol-replete conditions they are tightly bound to SREBP-cleavage activating protein (SCAP) via the regulatory COOH terminus. One role of SCAP is to "sense" the cellular content of cholesterol, which it does through its sterol-sensing domain (SSD), a polytopic intra-membrane region (Figure 1.8). When cholesterol is bound to the SSD of SCAP, another regulatory protein, Insig, binds the SCAP:SREBP complex and retains it in the ER membrane (Yang et al., 2002). However, when intracellular cholesterol levels fall and Insig releases SCAP, COP II coat proteins recognize a sequence in SCAP previously occluded by Insig, which causes the SCAP:SREBP to be incorporated into transport vesicles bound for the Golgi (Sun et al., 2007a). In the Golgi SREBPs are cleaved at two intra-membrane sites by distinct enzymes called Site-1 protease (S1P) and Site-2 protease (S2P). The action of the two proteases releases the NH_2-terminal bHLH-Zip domain of the SREBP, which enters the nucleus and binds target sequences, sterol regulatory elements (SRE), in the promoters of genes controlling lipid synthesis. Approximately 30 genes are activated by SRE, among

which are genes controlling cholesterol, fatty acid, triglyceride and phospholipid synthesis, and a few involved in glucose metabolism. Experiments in transgenic mice over-expressing just one type of nSREBP have shown that SREBP-1a and -1c act preferentially on fatty acid synthesis, whereas SREBP-2 action favours cholesterol synthesis (Brown and Goldstein, 1997), but *in vitro* both SREBP-1a and -2 can activate transcription of all the enzymes required for cholesterol synthesis with similar potency (Sakakura et al., 2001; Horton et al., 2002), suggesting that the transcription factors may be capable of compensating for loss in function. While SREBP-1a and -2 predominate in cultured cells, most tissues in the human body primarily express SREBP-1c and -2 (Horton et al., 2002). Each SREBP is assisted by co-regulatory factors. The presence of two co-regulators of SREBP-2, nuclear factor Y and cAMP response element-binding (CREB) protein, act synergistically to activate HMG-CoA reductase expression (Bennett and Osborne, 2000; Ngo et al., 2002; Woo et al., 2005).

Several classes of drugs are designed to take advantage of the transcriptional feedback mechanism that governs cholesterol biosynthesis. The statins are one such group. Statins are potent LDL-cholesterol-lowering drugs and they act by binding to HMG-CoA reductase and inhibiting conversion of HMG-CoA to mevalonate, thereby depriving the cell of *de novo* synthesized cholesterol. The cell then detects the decline in cholesterol and reacts by inducing the SREBP pathway. Therefore, the key to the mechanism of statins is the continual inhibition of HMG-CoA reductase and the accompanying *de novo* cholesterol synthesis, while at the same time SREBP-2 activates transcription of LDLR, which enhances cholesterol clearance from the plasma.

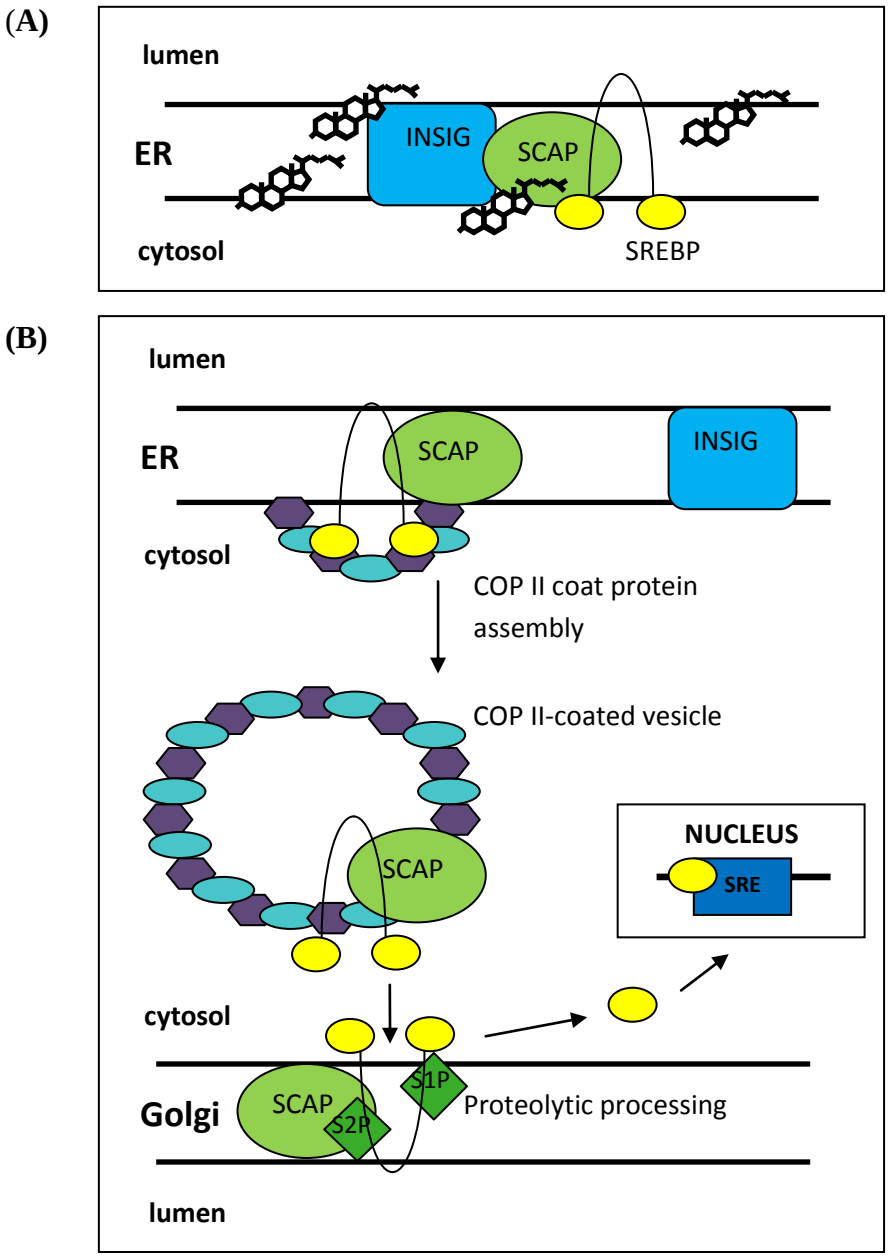

Figure 1.8 Transcriptional Regulation of HMG-CoA Reductase

(A) Under conditions of high cholesterol, cholesterol is bound to the sterol-sensing domain of SCAP. Insig binds the SCAP:SREBP complex and retains it in the ER membrane. (B) Under conditions of low cholesterol, Insig and SCAP do not interact, SCAP:SREBP is incorporated into COP II coated vesicles and transported to the Golgi, where S1P and S2P cleave the N-terminus of SREBP. The soluble SREBP enters the nucleus, binds sterol regulatory elements and activates gene transcription (McPherson and Gauthier, 2004).

1.2.3.2 Translational Regulation of HMG-CoA Reductase

The translational control mechanism for HMG-CoA reductase has not been as extensively studied as the transcriptional mechanism and is not fully understood. Translational regulation was investigated by the Goldstein-Brown laboratory and others in the 1980's, however, it soon became clear that the primary control mechanism was transcriptional, and so interest in the translational mechanism waned. Nevertheless, some key points are worth describing here. Initial experiments showed that HMG-CoA reductase could not be fully suppressed by sterols alone, since sterols predominantly reduce transcription from the HMG-CoA reductase gene. Even in the presence of maximal levels of sterols, cells retained enough of the reductase to synthesize small amounts of mevalonate, which was preferentially shunted into non-sterol pathways (Brown and Goldstein, 1980). When cells were treated with an HMG-CoA reductase inhibitor, thereby abolishing the synthesis of mevalonate entirely, the amount of enzyme increased even in the presence of sterols. Only upon addition of exogenous mevalonate together with HMG-CoA reductase inhibitor could the enzyme be completely suppressed. A study by Peffley and Sinensky (Peffley and Sinensky, 1985) in which HMG-CoA synthase deficient cells were used, showed that addition of mevalonate in the presence of sterols inhibited the translation of HMG-CoA reductase mRNA, and this finding was confirmed by Nakanishi et al. (Nakanishi et al., 1988). Mevalonate was shown to reduce the efficiency of translation of HMG-CoA reductase mRNA, but not by changing the amount of mRNA present. Instead, mevalonate accelerated degradation of the reductase protein, and it is believed that it did so either directly or as a non-sterol intermediate. It has been suggested that this negative regulation may take place at the translational level,

as some HMG-CoA reductase mRNA transcripts have complex 5'-untranslated regions, which may allow regulatory factors to act on these regions (Reynolds et al., 1985). The significance of this complex regulatory mechanism remains somewhat obscure, but it has been suggested that it may allow the cell to maintain a pool of HMG-CoA reductase mRNA that can be rapidly translated should mevalonate levels fall during a critical sterol-dependent process such as cell division (Nakanishi et al., 1988). The importance of maintaining a small amount of mevalonate for synthesis of non-cholesterol products is likely a factor. Nevertheless, on a day-to-day basis the translational mechanism likely has a lesser impact than the transcriptional and post-translational mechanisms do on maintaining cholesterol homeostasis.

1.2.3.3 Post-translational Regulation of HMG-CoA Reductase

Phosphorylation

Human HMG-CoA reductase is regulated post-translationally by reversible phosphorylation on Ser-872 (Beg et al., 1978). In liver, this modification takes place primarily via AMP-activated protein kinase (AMPK) (Carling et al., 1989) and serves to inactivate HMG-CoA reductase. Two other kinases, a Ca^{2+}/calmodulin-dependent protein kinase (CaMK) and protein kinase C have also been shown to be capable of phosphorylating HMG-CoA reductase at the same site (Beg et al., 1987). Of these three kinases, only AMPK has been demonstrated to play a physiologically important role in hepatic lipid metabolism. CaMK is activated upon increased intracellular calcium concentrations, and while this kinase is highly expressed in neural tissues, a role for calcium-dependent HMG-CoA reductase phosphorylation in liver remains to be investigated (Beg et al., 1986). Phosphorylation of HMG-CoA reductase by protein

kinase C is likewise regulated by calcium influx into the cell, as well as phospholipids, and has been demonstrated in rat brain isolates (Beg et al., 1985). Little is known about the role of PKC-mediated HMG-CoA reductase phosphorylation in the liver (Beg et al., 1987).

In most cases, the purpose of HMG-CoA reductase inactivation by post-translational modification is thought to be linked to conservation of ATP. AMPK acts as a sensor of cellular energy status that is conserved in all eukaryotic cells (Hardie, 2008). Under energy-depleted conditions, a high AMP:ATP ratio exists in the cell. AMPK is subsequently activated and promotes energy conservation and ATP production by affecting both the phosphorylation state of other proteins and their gene expression. Targets of AMPK include key enzymes involved in glucose metabolism, cholesterol synthesis, fatty acid oxidation and synthesis, protein synthesis, and cell growth and apoptotic pathways (Hardie, 2004). Both AMPK and HMG-CoA reductase are ultimately dephosphorylated by protein phosphatase 2A (Gaussin et al., 1997).

AMPK was initially identified by two separate groups, one studying the inhibitory effect of phosphorylation of HMG-CoA reductase and the other examining the effect on acetyl-CoA carboxylase (ACC), a key enzyme in the synthesis of fatty acids. The studies that showed HMG-CoA reductase and ACC were inactivated by an upstream kinase were both published in 1973 (Beg et al., 1973; Carlson and Kim, 1973). However, the discovery that the same kinase (AMPK) acted on both these enzymes occurred 15 years later (Carling et al., 1987). AMPK is thought to suppress these pathways under conditions of low ATP due to their high rate of energy consumption. AMPK itself is part of a protein signalling cascade (Figure 1.9). At the top of the cascade, AMP acts in two

ways to activate AMPK; it is an allosteric regulator, binding a site on the catalytic α-subunit of AMPK. In addition, AMP increases the affinity between AMPK and the kinase that phosphorylates it. This dual role of AMP allows cells to be extremely sensitive to small changes in AMP concentrations (Hardie and MacKintosh, 1992), which is particularly important in liver, where the activity of AMPK is relatively high. Recent studies have identified the major AMPK kinase as tumour suppressor LKB1, which phosphorylates AMPK on Thr-172 (Hawley et al., 2003; Shaw et al., 2004). Disruption of LKB1 results in the loss of AMPK activity, demonstrating the importance of this kinase in hepatic tissue (Shaw et al., 2005). Further downstream effects of AMPK include transcriptional control over several pathways, as shown in Figure 1.9. It has been reported that AMPK activation by adenosine analogue AICAR or by the use of adenovirus-mediated over-expression of a constitutively active form of the α2 catalytic subunit inhibits glycolytic and lipogenic gene expression (Foretz et al., 1998; Leclerc et al., 1998; Woods et al., 2000; Leclerc et al., 2001; Foretz et al., 2005), suggesting that AMPK acts to preserve glucose for ATP-producing pathways rather than for energetically expensive lipid synthesis. In addition, AMPK has been shown to reduce the expression of SREBP-1c, a transcription factor that plays a role in regulating lipogenic genes under the influence of insulin (Foretz et al., 2005).

Cyclic AMP (cAMP), cyclic GMP and glucagon have also been found to inhibit HMG-CoA reductase activity (Beg et al., 1973; Ingebritsen et al., 1979; Henneberg and Rodwell, 1985; Easom and Zammit, 1987). It has been suggested that these effects involve phosphorylation, although neither AMPK nor HMG-CoA reductase are substrates for cAMP-dependent kinase (Zammit and Easom, 1987). Instead, elevated cAMP levels

may help prevent dephosphorylation of HMG-CoA reductase, thereby promoting suppression of the reductase (Botham, 1992).

Degradation

A second post-translational mechanism of HMG-CoA reductase regulation is mediated by Insigs. Insigs were previously described as inhibitors of the SREBP pathway in the liver under conditions where lipids are sufficient. Insigs also mediate sterol-accelerated degradation of HMG-CoA reductase. HMG-CoA reductase contains a SSD (similar to that of SCAP) through which it monitors cholesterol levels in the cell. When the cell is replete with cholesterol, Insig associates with the cholesterol-bound SSD portion of HMG-CoA reductase, which recruits enzymes that ubiquitinate the reductase through a multi-step process and results in proteolytic degradation of HMG-CoA reductase by the proteasome. To date at least three ubiquitinases (U1, U2, and U3) have been shown to be involved (Pickart, 2001). Under conditions of low sterols, Insigs are not bound to HMG-CoA and degradation proceeds at a slower rate (Figure 1.8).

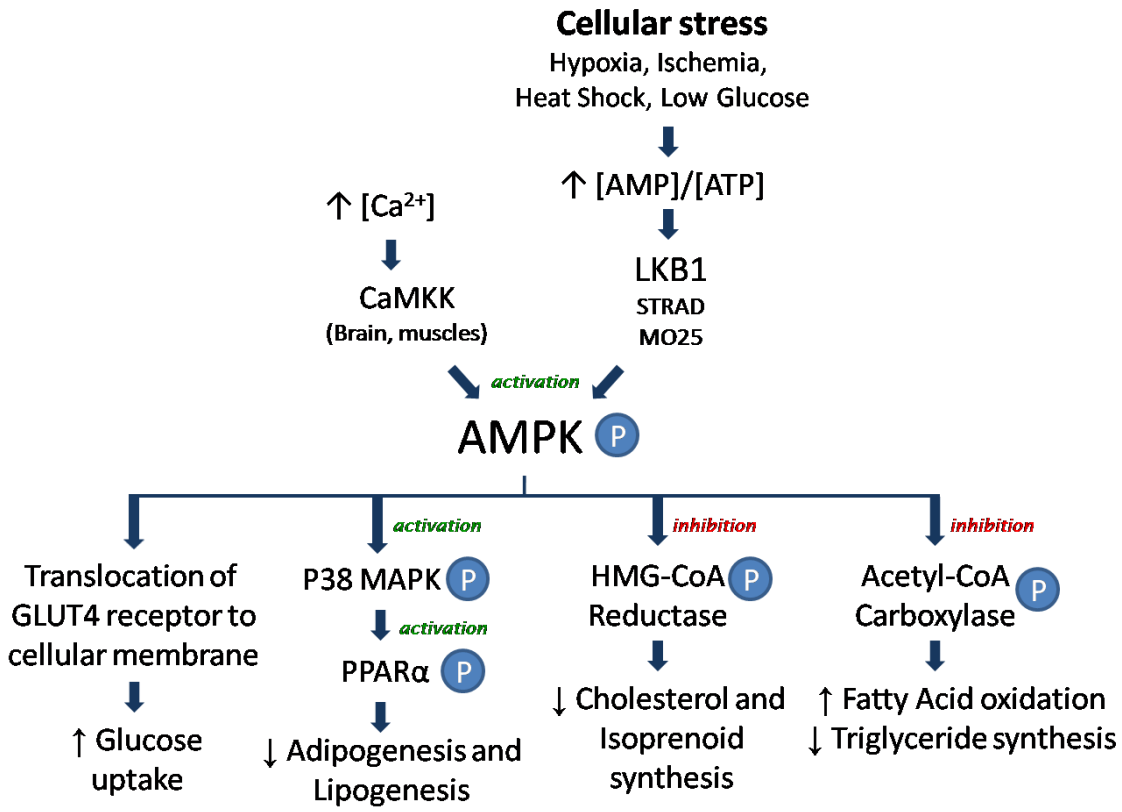

Figure 1.9 The AMPK Signalling Cascade

AMPK is an energy-sensing kinase that responds to increases in Ca^{2+} in some tissues and to increases in the AMP:ATP ratio. AMPK affects many downstream targets to bring about increases in ATP-generating processes and to suppress ATP-consuming pathways (Kahn et al., 2005; Towler and Hardie, 2007).
CaMMK, Ca^{2+}-calmodulin-dependent kinase kinase; STRAD, Ste20-related adaptor; MO25, Mouse protein 25

1.3 The Role of Natural Health Products in Atherosclerosis

1.3.1 A Definition of Natural Health Products

As defined by Health Canada, natural health products (NHP) include vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines such as traditional Chinese medicines, probiotics, and other products like amino acids and essential fatty acids. NHP are sold as over-the-counter products and do not require a prescription. Prior to marketing, all natural health products sold in Canada undergo a review to ensure that evidence supporting the safety, efficacy and quality of the product. The reviews are submitted to the Natural Health Products Dictorate, and those NHP that meet the requirements are issued a product licence and corresponding Natural Product Number that appears on the product label as proof that Health Canada has authorized the sale (Health Canada, 2009). Included under the broad definition of NHP are nutraceuticals, supplements combining elements of nutrition and pharmaceuticals. Agriculture and Agri-food Canada defines a nutraceutical as "a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with foods. A nutraceutical is demonstrated to have a physiological benefit or provide protection against chronic disease" (Agriculture and Agri-Foods Canada, 2008). Another group of nutritional/medicinal crossover products related to NHP are known as functional foods, characterized as products "similar in appearance to, or may be, conventional foods consumed as part of a usual diet, and demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions, i.e. contain one or more bioactive compound(s)" (Agriculture and Agri-Foods Canada, 2008).

1.3.2 History of Natural Health Product Use and Current Challenges

Natural health products have been in use for several thousands of years in the form of herbal and other plant-based medicines. Traditional Chinese medicine, which includes herbal medicines, acupuncture, food therapy and other methods, came into practice two to three thousand years ago. The original method of developing medicines was to test an unknown herb on oneself and observe the effects. Many healers died as a result of poisonous or noxious plants they ingested, but successful treatments were carefully documented and preserved for future generations (Guo et al., 2006). Ayurveda ("the science of life"), another system of traditional medicine native to the Indian Subcontinent and tied to Hindu religion, encompasses a similar range of practices, including herbs, surgery, massage and yoga. The overarching similarity between these and other forms of traditional medicine lies in their intrinsic aim to treat not only symptoms, but to address the cause of the malady and to consider the human body as a whole when applying treatment. Many herbal therapies in traditional medicine are a mixture of botanicals or extracts which include several components, rather than a single purified chemical. The complexity of the herbal mixtures is key to their effectiveness, as the components each play a specific and complimentary role in the body (Pang et al., 2002). In contrast, the focus of modern Western pharmacology is on single component drugs, which have the advantage of being strictly standardized and highly purified.

The relatively recent development of purified drug substances began in the 18th and 19th centuries, with isolation and identification of therapies such as aspirin (Bosch and Banos, 1998), morphine (Schmitz, 1985) and penicillin (Bennett and Chung, 2001). In developing countries, use of NHP may still be the major treatment for illness. The World

Health Organization reports that 80% of the world's populations rely on traditional medicines, primarily herbal in nature, in their primary healthcare (Chan, 2003). Most NHP have enjoyed a long history of safe and effective use, and many North Americans are currently taking some form of NHP on a daily basis. However, one cannot assume that "natural" is synonymous with "safe". Any substance taken in, whether natural or synthetic, has the potential for side effects and could be a health risk. In particular, there are concerns about interactions with prescription drugs, and mis- and self-diagnosis of medical conditions that may result in allergic reactions or overdose. Adverse effects, which can sometimes be life-threatening, have been associated with NHP contaminated with excessive or banned pesticides, heavy metals or chemical toxins (Koh and Woo, 2000; Chan, 2003). NHP are often regarded as low risk due to their long history of use, their natural origin, or the low dose required compared with many conventional drugs. However, all of these products have risk associated with them when combined in the body with other medications. Consumers must have access to products that are safe and effective, and to the information on how best to use them in combinations with other treatments. Health Canada is currently implementing regulation over NHP to ensure that the consumer has knowledge of the formulation and instructions for use (Health Canada, 2009).

1.3.3 An Example of Natural Health Product Therapy for Cardiovascular Disease: Omega-3 Polyunsaturated Fatty Acids

Omega-3 polyunsaturated fatty acids (PUFA) are an example of a NHP that has been proven to have significant benefits in treating CVD. Omega-3 PUFA are obtained in the diet through consumption of oily fish, such as herring, mackerel, salmon, tuna and

sardines, or by daily supplementation with fish oil capsules. The original sources of omega-3 PUFA in fish oil are the marine microorganisms the fish feed on.

Epidemiological data demonstrate an inverse relationship between fish consumption and cardiovascular mortality and morbidity (von Schacky, 2003). Numerous prospective and retrospective trials have shown that moderate consumption of omega-3 PUFA is beneficial in decreasing the risk of cardiovascular events, such as myocardial infarction, coronary heart disease, and death in patients with heart failure (Kromhout et al., 1985; Lavie and Milani, 1996; Albert et al., 1998; GISSI-Prevenzione Trial, 1999; Yokoyama et al., 2007; Lee et al., 2008; Tavazzi et al., 2008). Most of the benefits can be attributed to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two long-chain fatty acids in this group of PUFA. The American Heart Association currently endorses the use of omega-3 PUFA at a dose of approximately 1 g/day of combined DHA and EPA, either in the form of fatty fish or fish oil supplements (in capsules or liquid form) in patients with documented CHD (Kris-Etherton et al., 2002).

The primary action of omega-3 PUFA is to decrease triglyceride levels. It is believed that omega-3 PUFA bring about these effects through a reduction in VLDL secretion (Davidson, 2006). Omega-3 PUFA decrease fatty acid and triglyceride synthesis by inhibiting the activities of the liver X receptor and SREBP-1c, and increasing that of PPARα, thereby enhancing fatty acid oxidation. These actions deplete the hepatocytes' pool of triglycerides and promote increased degradation of apolipoprotein B, resulting in reduced output of VLDL particles. The actions of omega-3 PUFA also protect the endothelium (von Schacky, 2003) and inhibit platelet aggregation (von Schacky, 2006), both of which are factors that promote cardiovascular disease.

1.3.4 Reasons for Choosing a Natural Health Product to treat Hypercholesterolemia

The effectiveness of medications such as statins and cholesterol absorption inhibitors in reducing risk of cardiovascular events (i.e. stroke and myocardial infarction) is undeniable. Together, rosuvastatin/ezetimibe or simvastatin/ezetimibe can achieve significant reductions in LDL-C and alter cardiovascular endpoints (Daskalopoulou and Mikhailidis, 2006; Farnier et al., 2009; Polis et al., 2009). However, the widespread use of these drugs has shown that they are not universally well tolerated. A growing number of patients are statin adverse (Bruckert et al., 2005; Stein et al., 2008), and even low-dose statin treatment cannot be tolerated, which creates difficulty in reaching the blood lipid goals outlined by the National Cholesterol Education Program. It has been shown that statin-related adverse effects correlate with drug dose (Bays, 2006; Jacobson, 2006). In the first two decades of statin development and use, statin toxicity was viewed as a rare event. However, muscle-related side effects (MRSE) have become major impediments to instituting successful lipid-lowering therapy, with 5-10% of statin-treated patients experiencing MRSE (Venero and Thompson, 2009). Clearly, alternative therapies are needed to reduce cardiovascular risk and reinstate lipid balance in hypercholesterolemic patients.

In the past several decades, there has been increasing acceptance and recognition of traditional Chinese medicine and natural health products as effective treatments for various pathological conditions in Europe and North America (Siow et al., 2005). There are many reasons for this change in attitude. First, the concept of receiving a natural therapy is certainly appealing to many patients. Additionally, natural health products

tend to cause fewer side effects than synthetic medications, because of their often complex makeup and multiple modes of action in the body (Siow et al., 2005; Schmidt et al., 2008). Natural medications in the form of a mixture are also potentially more effective than synthetic chemicals, even when those chemicals are natural in origin (Pang et al., 2002). However, difficulties arise in obtaining standardized combinations, since natural products, particularly herbs, are highly variable depending on geographical origin, climate conditions and many other variables. The stricter guidelines currently being implemented in Canada for better NHP consumer protection are an important step in regulating the quality and consistency of these medications, and will help to ensure that promised health benefits are obtained.

1.3.5 Berberine as a Regulator of Hepatic Lipid Metabolism

Berberine is an isoquinolone alkaloid derived from the roots, rhizomes, and stem bark of plants such as barberry species (*Berberis vulgaris*), the Chinese herb *Huanglian* (*Coptis chinensis*) and goldenseal (*Hydrastis canadensis*), an herb native to eastern regions of North America. Extracts and decoctions from these plants have been used in Chinese and Ayurvedic medicine for many years as treatment for gastrointestinal disorders, liver dysfunction, gallbladder disease and urinary tract infections (Gruenwals, 1998; Jellin et al., 2000). Berberine extracts have been shown to have antimicrobial activity against a variety of organisms, including bacteria, fungi, viruses, protozoans and helminths. In China, berberine is predominantly used to treat bacterial diarrhea and intestinal parasite infections (Birdsall and Kelly, 1997). The structure of berberine is shown in Figure 1.10. Little is known about the pharmacokinetics of berberine and the details of its metabolic pathways are currently under investigation. It is known that berberine is absorbed from

the small intestine, and rapidly and extensively metabolized by the liver (Shen, 1993). Since the urinary metabolites detected after oral administration of berberine were the same as those detected after injection, it is believed that berberine is metabolized post-absorption, not in the small intestine (Qiu et al., 2008). Studies suggest that berberine is modified by hepatic cytochrome P450 enzymes, and undergoes phase I methylation and phase II glucuronidation; several of the phase II metabolites have been identified in the circulation and in rat and human urine (Budzinski et al., 2000). A study by Zuo and colleagues revealed four main metabolites of berberine and their glucuronide conjugates in rat plasma (Zuo et al., 2006). Due to rapid clearance of berberine from the circulation and metabolism by the liver, the authors concluded that the berberine metabolites, rather than berberine itself, may be the bioactive components. Another group designed a berberine derivative, dihydroberberine, with the aim of improving the bioavailability. The derivative was subsequently detected in plasma after a low dose was consumed orally by rats (Turner et al., 2008). The investigation into the actions of berberine metabolites is ongoing. Excretion of berberine and its derivatives occurs via both biliary and renal routes. Very little berberine is excreted without having first been metabolized (Tsai and Tsai, 2002). A number of studies demonstrate that berberine can penetrate the blood-brain barrier and it is currently being investigated for its neuroprotective properties (Kulkarni and Dhir, 2009).

Berberine has begun to gain attention from the medical community as a natural health product. Berberine has been shown to have beneficial effects in inflammation, obesity, diabetes and cancer (Ckless et al., 1995; Iizuka et al., 2000; Kim et al., 2008a; Yin et al., 2008b; Kim et al., 2009). A recent study has demonstrated berberine's protective

properties in macrophages under conditions of oxidative stress (Sarna et al., 2010). In addition, berberine has been shown to possess potent lipid-lowering effects. Berberine influences the cholesterol metabolism regulatory system in a different manner than the drugs commonly used to lower lipids, and its potential as a new treatment for hypercholesterolemia is under investigation. Two distinct mechanisms of action for berberine's lipid-lowering effects have thus far been described. The first lipid-lowering mechanism occurs through stabilization of LDLR mRNA, leading to improved LDL clearance from the circulation, and affects primarily LDL-C levels. The second mechanism occurs through activation of AMPK, resulting in inhibition of fatty acid synthesis and a decline in plasma triglycerides. These pathways are described in more detail below.

Berberine's ability to lower cholesterol was first shown to occur through the stabilization of LDLR mRNA, leading to an increase of LDLR on the hepatocellular membrane and enhanced clearance of LDL-cholesterol (Kong et al., 2004). The actions of berberine were shown to be independent of intracellular cholesterol levels and had no effect on SREBP promoter activity, indicating that SREBP are not involved in the mechanism of berberine-mediated LDLR up-regulation. Regulatory sequences involved in the turnover rate and the stability of LDLR mRNA were identified in the 3' untranslated region of the transcript, and they appeared to play an important role in the stabilization of LDLR mRNA by berberine (Kong et al., 2004). Another study identified two RNA binding proteins through which berberine may regulate LDLR mRNA stability (Li et al., 2009). Numerous studies have also indicated that activation of the MAPK/ERK pathway is a prerequisite event in berberine-mediated stabilization of the LDLR transcript (Kong et

al., 2004; Abidi et al., 2005; Brusq et al., 2006). Kong et al. demonstrated these events in human hepatoma (HepG2) cells and diet-induced hyperlipidemic hamsters. Additionally, a small clinical trial in hyperlipidemic patients given oral berberine supplements (0.5 g of berberine orally twice per day for 3 months) yielded decidedly promising results, with significant decreases in serum total cholesterol (29%), triglycerides (35%) and LDL-C (25%) , but no change in cardioprotective high density lipoprotein-cholesterol (Kong et al., 2004). In contrast to berberine's LDLR mRNA stabilizing role, statins act on HMG-CoA reductase to inhibit cholesterol synthesis and upregulate LDLR gene expression. While this mechanism explains the role of berberine in lowering serum cholesterol, it does little to account for the decline in triglycerides observed in human subjects. Hypertriglyceridemia contributes to a number of metabolic diseases, including cardiovascular disease, metabolic syndrome and type II diabetes (Cannon, 2008).

A second mechanism by which berberine lowers lipids, particularly triglycerides, was described by Brusq et al (Brusq et al., 2006). The authors showed that berberine activated AMP-activated protein kinase (AMPK), a kinase known to be involved in regulating energy balance in the cell (Hardie, 2008). The two primary targets of AMPK are acetyl-CoA carboxylase (ACC) and HMG-CoA reductase (Carling et al., 1989). Berberine caused AMPK phosphorylation, a finding which is supported by several other studies (Lee et al., 2006; Turner et al., 2008), and the discovery that activation of AMPK by berberine was MAPK/ERK-dependent was confirmed (Kong et al., 2004; Brusq et al., 2006). The subsequent phosphorylation of ACC by AMPK slowed triglyceride synthesis and promoted oxidation of fatty acids (Hardie and Pan, 2002). Several studies have

demonstrated the significant plasma triglyceride-lowering effect of berberine in humans since this phenomenon was initially observed (Yin et al., 2008b; Zhang et al., 2008).

A recent study measured the effect of a combination of berberine and simvastatin as cholesterol-lowering therapy in rodents and humans (Kong et al., 2008). The combination therapy decreased human and rat plasma LDL-cholesterol more effectively than either treatment alone, and boasted the advantage of requiring a low dose of both berberine and statin to achieve significant results. A significant reduction in plasma triglycerides was also observed.

In conclusion, berberine is an attractive candidate for lipid-lowering therapy. Its unique and multiple modes of action make it a good alternative to currently available treatments, and it may be of use in combination therapy. Berberine was well tolerated by clinical trial participants in lipid-lowering studies (Kong et al., 2004; Kong et al., 2008; Yin et al., 2008b; Zhang et al., 2008). Berberine has been shown to have positive effects on wide-ranging aspects of human health, playing a role in the regulation of metabolic disorders such as diabetes and metabolic syndrome, mental health disorders and cancer (Figure 1.11). Thus, further research into berberine's beneficial health effects is certainly warranted.

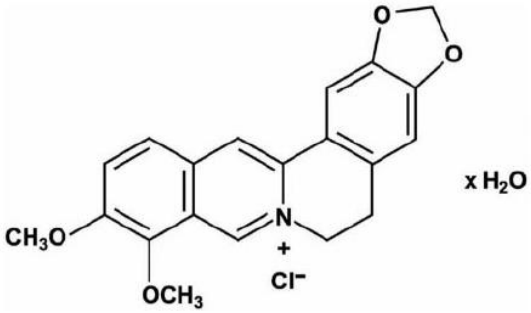

Figure 1.10 The Chemical Structure of Berberine.

Berberine is an isoquinoline alkaloid derived from the roots, rhizomes and stem bark of various plants. It is vividly yellow coloured and was used traditionally as a dye for leather, wool and wood. It also has anti-microbial activity, and its role in cardiovascular conditions and inflammation is under investigation.

This image is in the public domain.

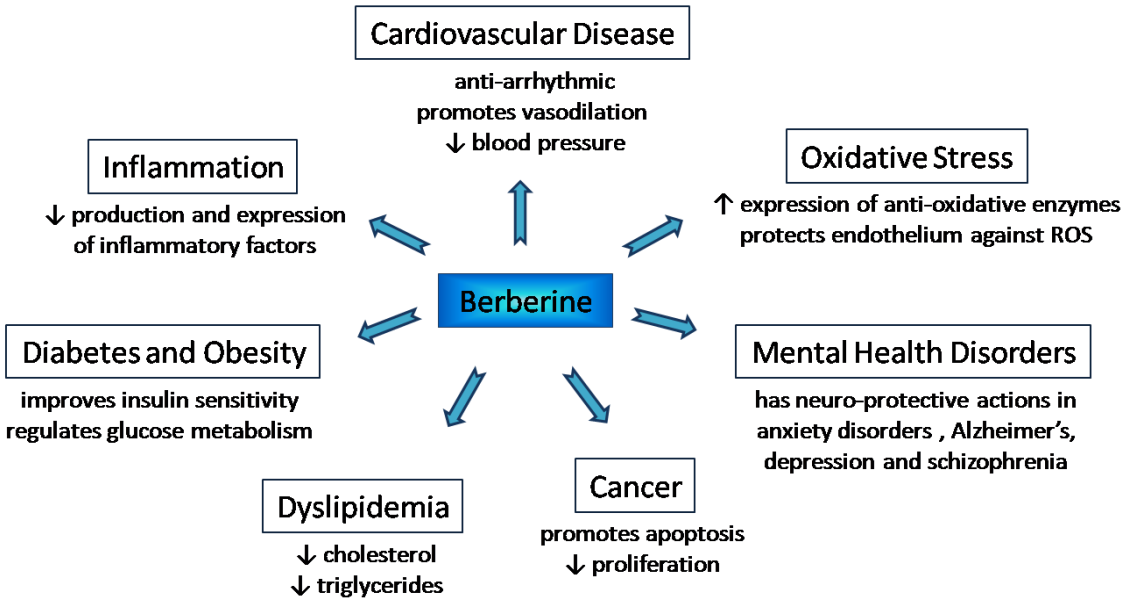

Figure 1.11 Berberine's beneficial effects in different disease states.

Berberine has been shown to have many roles in improving health. Studies suggest berberine has benefits in cardiovascular disease (Lau et al., 2001), diabetes and obesity (Lee et al., 2006; Kim et al., 2009; Zhang et al., 2009), dyslipidemia (Kong et al., 2004; Brusq et al., 2006), inflammatory disease (Ckless et al., 1995; Fukuda et al., 1999b; Jeong et al., 2009), oxidative stress (Liu et al., 2008a; Liu et al., 2008b), cancer (Fukuda et al., 1999a; Lin et al., 2006; Tang et al., 2009) and mental disorders (Zhu and Qian, 2006; Kulkarni and Dhir, 2009).

Hypothesis and Objectives

Hypothesis

Our lab is investigating natural health products as potential treatment for cardiovascular disease such as atherosclerosis. Recently, we have established the role of homocysteine in the activation of HMG-CoA reductase and the benefits of folic acid supplementation in cell and animal models (Woo et al., 2005; Woo et al., 2006a; Woo et al., 2006b). Our study in a macrophage cell line has demonstrated the protective effects of berberine under conditions of oxidative stress (Sarna et al., 2010). We believe that berberine has great potential as a natural therapeutic agent as it has already been shown to have many beneficial effects on the dyslipidemic condition (Lee et al., 2006; Zhao et al., 2008; Zhou et al., 2008). In this project, we chose to investigate whether berberine might have an effect on HMG-CoA reductase activity based on the preliminary results of our screening process for new lipid metabolism regulators. We hypothesized that berberine would inhibit HMG-CoA reductase and decrease cholesterol synthesis in hepatocytes.

Objectives

The objectives of this study were to determine (1) the effect of berberine on cholesterol biosynthesis in hepatocytes; (2) the mechanism by which berberine regulates HMG-CoA reductase activity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

See Appendix I

2.1.2 Buffers

See Appendix II

2.1.3 Equipment

See Appendix III

2.2 Cell Model System

The HepG2 human hepatoma cell line (American Type Culture Collection, Manassas, VA, USA) has been used extensively in studies of cholesterol synthesis and metabolism. While results obtained from immortalized cell lines should be interpreted with caution, it has been shown that the metabolic pathways studied here regarding cholesterol metabolism are intact and functional in HepG2 cells (Javitt, 1990; Gibbons, 1994), and so these cells provide a valuable model system in which to study lipid metabolism. HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Waltham, MA) with 10% foetal bovine serum (FBS; Hyclone) at 37°C with 5% CO_2. The cells were seeded and allowed to attach for 8 hrs, then cultured overnight (13-16 hrs) in DMEM without FBS. Treatment with berberine occurred the morning after overnight serum deprivation at 80-90% at cell confluency. Berberine was prepared at a stock concentration of 50 mM in DMSO and stored at -20°C. The cells were treated with concentrations of berberine ranging from 25-200 μM for various time periods.

Cell culture in serum-free medium is known to induce changes in the activity and expression of many enzymes. Serum contains numerous hormone factors that stimulate growth and differentiation, transport proteins that carry minerals, vitamins and lipids, and many other factors that affect cell stability or toxicity (van der Valk et al., 2004). Serum deprivation obliges the cells to enter a quiescent stage in which there is minimal signalling activity and growth, and is one method for synchronizing cellular growth for cell cycle studies (Davis et al., 2001).

In this study, serum deprivation is used to up-regulate HMG-CoA reductase activity. The exclusion of foetal bovine serum (FBS) from the cell medium deprives the cells of many nutrients, including lipoproteins. As a result, the cells are forced to synthesize their lipoprotein components, including cholesterol, in order to continue growing and dividing. Cholesterol is an important structural element of the cell membrane and is essential for cell division. Prolonged serum deprivation eventually leads to apoptosis, as the cells are slowly starved of nutrients. For HepG2 cells, the time required to induce apoptosis by serum deprivation is 72-96 hrs (Takehara et al., 2001; Wu et al., 2002; Kang et al., 2003). In this study, the longest period of serum deprivation used is 24 hrs. As shown in Figure 2.1, serum deprivation resulted in a time-dependent increase in HMG-CoA reductase activity in HepG2 cells. After 14 hrs of serum deprivation, the cells had HMG-CoA reductase activity that was about 3.5-fold higher than cells that had not been serum deprived. All experiments were conducted with cells stimulated in this manner unless otherwise indicated.

A model of stimulated HMG-CoA reductase activity is comparable to the high HMG-CoA reductase activity observed in individuals suffering from heterozygous familial hypercholesterolemia (Hoeg et al., 1986; Hopkins, 2003). Due to the inability to take up LDL-C from the plasma efficiently, the negative feedback system fails and hepatic cholesterol synthesis occurs at an elevated rate in these individuals. The resulting high levels of circulating cholesterol are due in part to the high activity of HMG-CoA reductase coupled with the reduced ability to take up LDL via the LDL receptor. Although reducing HMG-CoA reductase activity does not fully solve the root problem in familial hypercholesterolemic patients, it does bring lipid levels down, and thereby

decreases the risk for development of atherosclerosis and cardiac events. In the present study, serum-deprived HepG2 cells serve as a model for an individual with elevated HMG-CoA reductase activity.

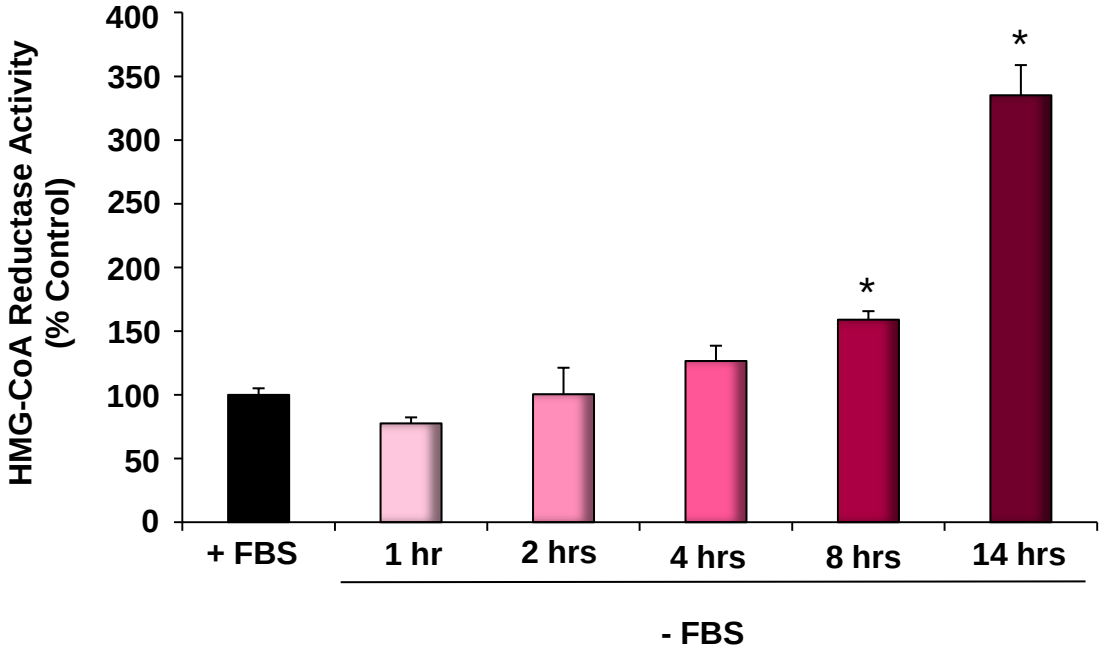

Figure 2.1 The Effect of Serum Deprivation on HMG-CoA Reductase Activity in HepG2 Cells

HMG-CoA reductase activity was compared in HepG2 cells cultured in DMEM + 10% FBS (+FBS, taken as control in this experiment) and in cells that were serum-deprived (-FBS) for various time periods. The percent change in activity as compared to control was calculated. Results were expressed as mean ± SEM.

2.3 Experimental Methods

2.3.1 Enzyme Activity Assays

2.3.1.1 Determination of Enzyme Activity in Cell Lysate

The activity of HMG-CoA reductase was measured by tracking the conversion of radioisotope-labelled HMG-CoA (Wilce and Kroon, 1992). Cells were incubated in the presence or absence of berberine, rinsed with ice-cold PBS, and collected by scraping in cell lysis buffer (50mM Tris-HCl, 150mM NaCl). The cells were pelleted and stored at -80°C overnight. The cell pellet was dissolved in phosphate buffer 1 (50 mM K_2HPO_4, 5 mM DTT, 1 mM EDTA, pH 7.4) and the protein concentration was determined by the Bradford method using BSA as a standard. To 50 ug protein was added phosphate buffer 2 (100 mM K_2HPO_4, 6 mM DTT, 1.2 mM EDTA), with final concentrations of 20 mM glucose-6-phosphate, 2.5 mM NADPH, 1 U glucose-6-phosphate dehydrogenase; Sigma-Aldrich), and the samples were incubated for 15 min in a waterbath (Isotemp 205, Fisher Scientific) at 37°C. The substrate (8 Ci/mol, 1.0 mM [3-^{14}C]HMG-CoA; PerkinElmer Life Sciences, Boston, MA) was added to a final concentration of 50 uM and the samples were incubated for 30-60 min at 37°C. The reaction was terminated by adding 10 μL 5 M HCl, and 1 mg mevalonolactone was included as an internal marker, after which another incubation of 30 min at 37°C followed. The samples were then pelleted by centrifugation (Centrifuge 5804R, Eppendorf), and the supernatant was collected, spotted on a silica gel TLC plate (EMD Chemicals Inc., Gibbstown, NJ) and run in solvent consisting of chloroform:acetone (2:1 v/v). The separated bands were visualized with iodine staining and cut from the TLC plate, dissolved in toluene (EMD Chemicals Inc.) and counted with a scintillation counter (Beckman Coulter LS 6500).

HMG-CoA reductase activity was calculated and expressed as pmol of mevalonolactone produced per mg of protein per minute, and as % control. Cells that had been serum-deprived, but had not been treated with berberine, were used as a control in this and all following experiments. The product measured, mevalonolactone, is a derivative of mevalonate (the product of the reaction HMG-CoA reductase catalyzes). Mevalonolactone is created during the acidification step and is effectively separated from HMG-CoA during the following thin layer chromatography procedure.

2.3.1.2 Determination of Enzyme Activity in Cell Lysate Incubated with Berberine

In order to determine whether berberine was having its inhibitory effect by binding directly to HMG-CoA reductase, cell lysate was incubated with berberine *in vitro*, and enzyme activity was then assessed. *In vitro* assay conditions differed in the following steps: cells were not treated with berberine before collecting, but were lysed and frozen overnight. To begin the assay, the cell pellets were dissolved in buffer 1. Berberine was added directly to the cell lysate for 30 min at 37°C, then the assay commenced with the addition of reaction buffer 2 as above.

2.3.2 Cell Viability Assay

Cell viability was assessed using a colorimetric assay with 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT; Bio Basic Inc, Markham, ON) (Mosmann, 1983). Cells were seeded in 96-well plates at a density of $1.0x10^4$ cells per well and cultured in the same manner as described above (8 hrs growth in DMEM + 10% FBS, overnight in DMEM alone), then treated with berberine for 6 hrs. After treatment, cells were incubated for 4 hrs in the presence of MTT (0.5 mg/ml). This method is based on the conversion of MTT to MTT-formazan crystals by mitochondrial enzymes. The

reduction of MTT to MTT-formazan occurs only in viable cells. MTT-formazan was extracted with DMSO and the optical density of formazan (540 nm) was detected using a plate reader (SpectraMax M5, Molecular Devices). A reduction in optical density compared with control cells indicates a loss of cell growth and viability.

2.3.3 Filipin Staining of Free Cholesterol

2.3.3.1 Fluorescence Microscopy and Image Capture

Cells were seeded on sterile glass coverslips placed in 35 mm cell culture. The cells were treated with berberine, washed 3x with sodium buffer, fixed for 20 min with 10% formaldehyde and washed again 2x with sodium buffer. Filipin (Filipin complex from Streptomyces filipinensis, Sigma-Aldrich) was dissolved in DMSO at 1 mg/ml and diluted in sodium buffer to 50 μg/ml, and added to the cells, which were incubated in the dark for 2 hrs at room temperature. The stained cells were washed 3x with sodium buffer. After staining, the coverslips on which the stained cells were fixed were carefully removed from the plates and mounted on glass slides using 80% glycerol as adherent. The slides were immediately viewed on a fluorescence microscope (Zeiss Axioskop MOT) and photographed using a digital camera (Axiocam). Accurate measurement of fluorescent intensity by analysis of the photos obtained by this method was not possible due to rapid photobleaching of the stain. However, the photos are included to visually show the cholesterol in the cells.

2.3.3.2 Measurement of Fluorescent Intensity

In order to obtain an accurate measurement of fluorescent intensity, cells were plated in a black-bottomed 96-well plate at a density of $2x10^4$ cells/well. The treatment, cell fixing and staining procedures were identical to those described above. The fluorescent

intensity was measured immediately using a SpectraMax M5 plate reader (Molecular Devices) with the excitation wavelength set at 350 nm and emission at 420 nm.

2.3.4 Real-time Polymerase Chain Reaction (PCR)

2.3.4.1 Isolation of Total RNA

Cells were treated with berberine as described above, washed 3x with PBS and collected with TRIzol reagent (Invitrogen, Carlsbad, CA) to isolate total RNA. The TRIzol-dissolved cell pellet was gently mixed with 200 μL chloroform. The aqueous layer was collected after centrifugation at 12,000 x *g* for 15 min at 4°C, and RNA was precipitated by the addition of 500 μL isopropanol (Fisher Scientific). The RNA pellet was once again centrifuged at 12,000 x *g* for 15 min at 4°C, and washed with 75% ethanol in sterile DEPC-treated water. A final centrifugation at 12,000 x *g* for 10 min at 4°C followed. Finally, the ethanol was removed and RNA pellet was dissolved in sterile DEPC-treated water. The concentration of RNA was measured by spectrophotometry.

2.3.4.2 Real-time PCR

HMG-CoA reductase mRNA expression was determined by quantitative real-time PCR analysis using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). 2 ug total RNA was converted to cDNA by reverse transcription. The real-time PCR reaction mixture contained 0.4 uM hHMGR 5'-ACCAAGAAGACAGCCTGAA-3' (Forward) primer, hHMGR 5'-CATCCTCCACAAGACAATGC-3' (Reverse) primer (GenBank accession No. NM_000859), and 2 ul cDNA product in iQ-SYBR Green supermix reagent (Bio-Rad). Threshold crossing values were normalized to GAPDH (primer sequences 5'-TCAAGAAGGTGGTGAAGCAG-3' (Fw) and 5'-AGGTGGAAGAATGGGAGTTG-3' (Rev); GenBank accession No. NM_017008). The

mRNA expression was expressed as percentage change relative to control. All primers were purchased from Invitrogen.

2.3.5 Western Immunoblotting Analysis

Cellular levels of specific proteins were determined by Western blot analysis (Laemmli, 1970). After treatment, cells were lysed with protein lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2.1 μM leupeptin, 1 mM PMSF, 1% Triton-X 100). Protein was equalized to 40 ug in each sample, then denatured and separated by electrophoresis on a 10% SDS polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane, which was blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 hr at room temperature. After washing 3x for 10 min with TBST, the membrane was probed with primary antibodies for the target proteins overnight at 4°C. The primary polyclonal antibodies used were anti-HMG-CoA reductase (Upstate), anti-phospho-HMG-CoA reductase (Upstate), anti-AMPKα (which detects both α1 and α2 isoforms, but not β or γ subunits; Cell Signalling Technology), and anti-phospho-AMPKα (Cell Signalling Technology). The phospho-HMG-CoA reductase antibody binds specifically to the phosphorylated Ser-872 residue, and the phospho-AMPK antibody recognizes phosphorylation at the Thr-172 site only. β-Actin, a standard "housekeeping gene", was used as an internal standard in all immunoblotting assays. The following day, the membrane was incubated with peroxidase-conjugated secondary antibody for 1 hr at room temperature. Bands corresponding to target proteins were visualized with enhanced chemiluminescence (ECL) reagents using hydrogen peroxide as a substrate. The ECL signal was detected by

X-OMAT blue film (Kodak). The film was scanned and converted to a tiff file, and the bands were analyzed with a gel documentation system (Bio-Rad Gel Doc 1000) and Quantity One software version 4.2.1.

2.3.6 Statistical Analysis

The results were analyzed using a two-tailed independent Student's t-test. The level of statistical significance was set at $p<0.05$; all experiments were performed in duplicate or triplicate (n=10 to 14). Error bars denote the mean ± SEM.

Chapter 3

Results

3.1 Identification of Berberine as an Inhibitor of HMG-CoA Reductase and Investigation of its Effect on Cellular Cholesterol Levels

The first objective of this study was to identify a natural health product that is capable of regulating HMG-CoA reductase. Several compounds were screened for their ability to inhibit HMG-CoA reductase activity. Berberine was one of these, and it showed potential as a regulator of HMG-CoA reductase. We further investigated the ability of berberine to inhibit cholesterol synthesis.

3.1.1 Optimization of the HMG-CoA Reductase Activity Assay

The optimal conditions for the HMG-CoA reductase activity assay were determined by creating protein concentration curves (Figure 3.1). A range of protein (20 – 100 μg) was tested to establish the maximum specific activity. A linear relationship between the amount of cellular protein and enzyme activity (pmol product/min) was observed. Enzyme activity was converted to specific enzyme activity (pmol product/min/mg protein), and the optimal protein range was determined to be 30 – 40 μg, since the highest specific activity was achieved using this amount of protein. This range was used in all subsequent HMG-CoA reductase activity assays.

3.1.2 Effect of Berberine on HMG-CoA Reductase Activity

The effect of berberine on HMG-CoA reductase activity was assessed in HepG2 cells. Cells were cultured overnight in DMEM with or without 10% FBS and treated with berberine the following day (100 μM berberine for 6 hrs). Cells stimulated by culturing in serum-deprived DMEM overnight exhibited high HMG-CoA reductase activity, which was partly alleviated by treatment with berberine (Figure 3.2). The activity of HMG-CoA reductase in cells treated for 6 hrs with 100 μM berberine were decreased by about

27% below control (cells that had not been treated with berberine). It was noted that berberine also decreased HMG-CoA reductase activity in un-stimulated cells. Next, different concentrations of berberine were used to treat the stimulated cells. Treatment with berberine (25-100 μM) for 6 hrs diminished HMG-CoA reductase activity in a dose-dependent manner (Figure 3.3). A maximum decrease of approximately 30% below control was observed after 6 hrs treatment of 100 μM berberine. The inhibitory effect of 100 μM berberine on HMG-CoA reductase was time-dependent (Figure 3.4), seen initially after 6 hrs of treatment with a decrease of 27% and maintained even after 24 hrs treatment.

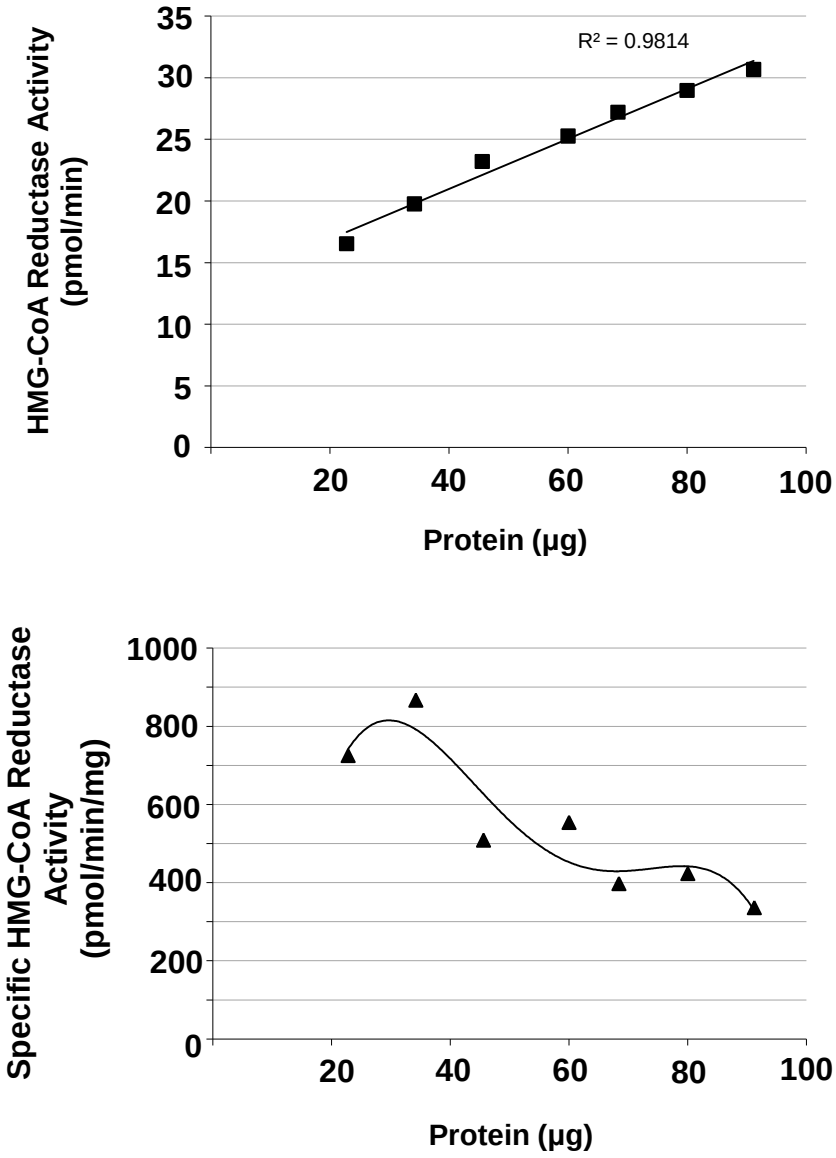

Figure 3.1 Optimization of the HMG-CoA Reductase Activity Assay in HepG2 Cells

HepG2 cell protein (no berberine treatment) was collected in cell lysis buffer. A range of protein concentrations was tested in the HMG-CoA reductase activity assay. Results are expressed as enzyme activity (pmol/min) and specific enzyme activity (pmol/min/mg protein). The optimal amount of protein required to achieve maximum specific HMG-CoA reductase activity was determined to be 30 – 40 µg.

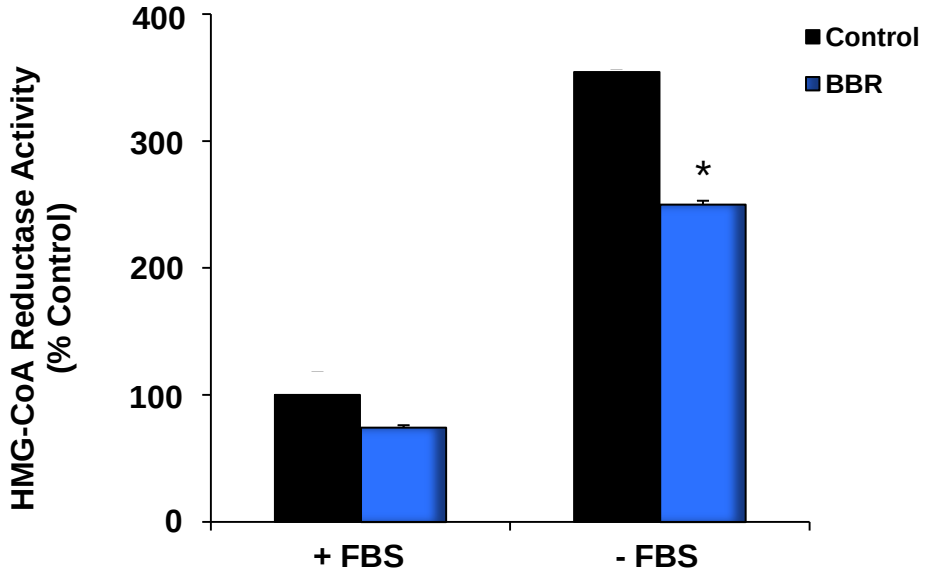

Figure 3.2 The Ameliorative Effect of Berberine on Elevated HMG-CoA Reductase Activity in Serum-Deprived HepG2 Cells.

HepG2 cells were cultured in DMEM +10% FBS (+FBS) or in DMEM containing no FBS (- FBS) overnight. HMG-CoA reductase activity was determined after cells were incubated for 6 hrs in the absence (control) or presence of berberine (BBR, 100 μM).

*p<0.01 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

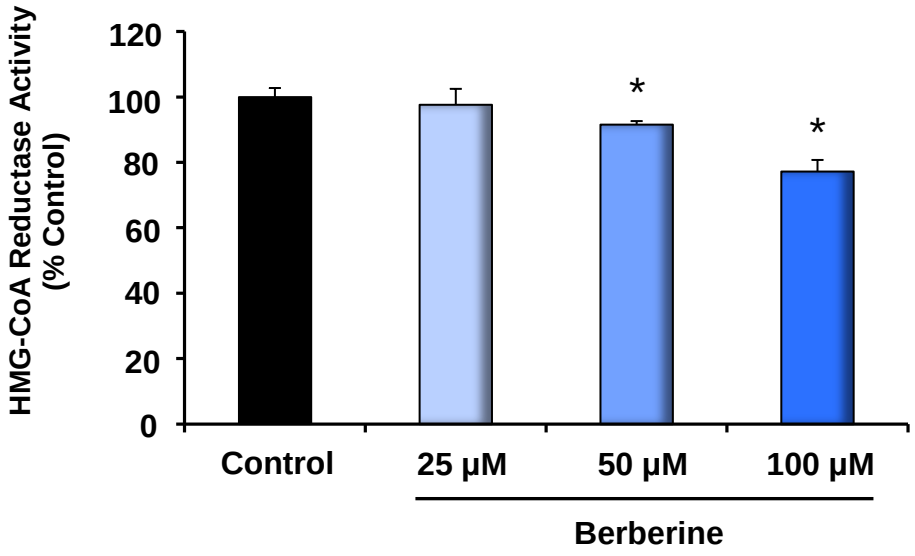

Figure 3.3 The Dose-dependent Effect of Berberine on HMG-CoA Reductase Activity

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation. HepG2 cells were then treated with berberine (25-100 µM) for 6 hrs and HMG-CoA reductase activity was determined.

*p<0.001 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

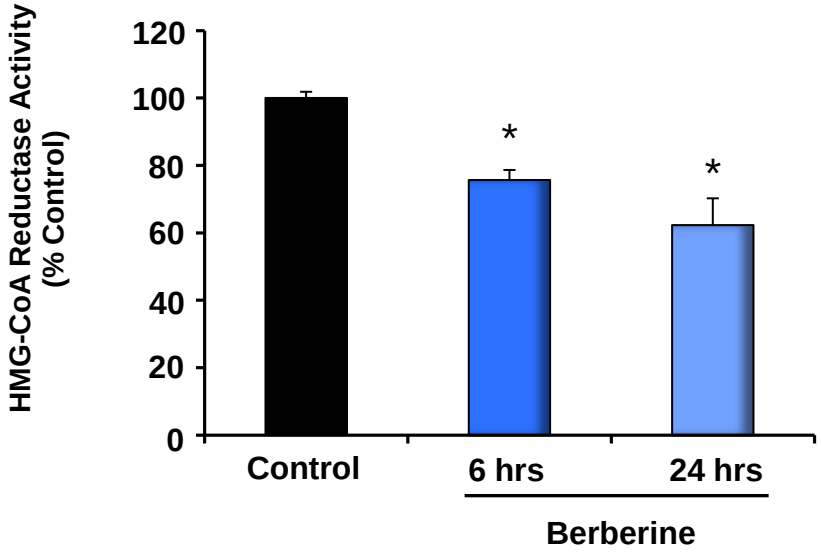

Figure 3.4 The Time-dependent Effect of Berberine on HMG-CoA Reductase Activity

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum deprivation. The following day, the cells were incubated in the absence (control) or presence of 100 μM berberine for various time periods and HMG-CoA reductase activity was measured. The specific activity of HMG-CoA reductase in control cells was similar at all time points after 14 hrs of serum deprivation.

*p<0.05 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

3.1.3 Effect of Berberine on Cell Viability

Berberine demonstrated low toxicity in virtually all participants of clinical trials. The toxic effect of berberine in our cell model was assessed by performing a cell viability assay. HepG2 cells were deprived of serum overnight and treated for 6 hrs with berberine (25-200 μM), at which time MTT was added to the culture medium. Viable cells take up MTT and convert it to formazan, which is solubilised by the addition of DMSO. Optical density measurements are taken to determine the number of viable cells. Optical densities for cells treated with 100 μM berberine were not significantly different from control (Figure 3.5). This concentration was used in all subsequent experiments.

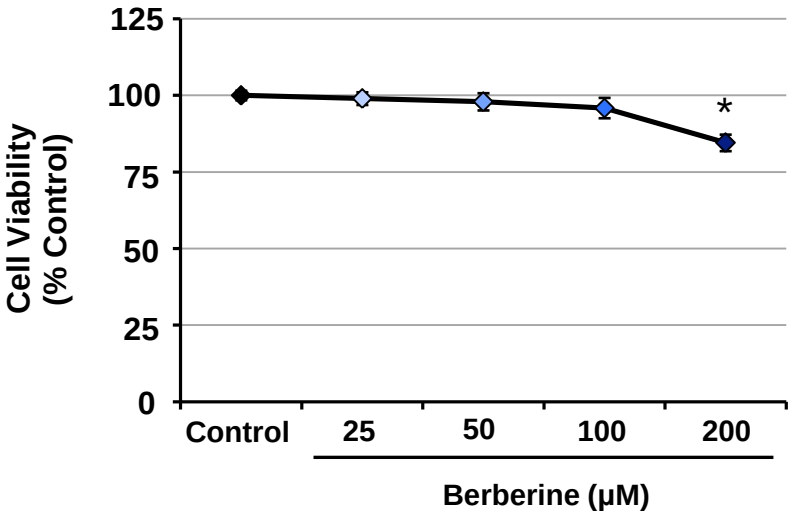

Figure 3.5 The Effect of Berberine on Cell Viability

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation. The following day, the cells were treated with various concentrations of berberine for 6 hrs, after which cell viability was assessed. No significant change in cell viability was detected after 6 hrs treatment with 100 μM berberine. This was the concentration used in all subsequent experiments.

*p<0.05 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

3.1.4 Determination of Intracellular Cholesterol Levels

In order to determine intracellular cholesterol levels, HepG2 cells were stained with filipin, a fluorescent dye molecule that binds neutral un-esterfied cholesterol. Traditional methods of cholesterol measurement, such as lipid extraction and colorimetric assays, were not possible due to the yellow colour from berberine treatment present in the sample, which interfered with spectrophotometric readings. No such difficulties occurred during staining with filipin, because berberine did not fluoresce under the excitation wavelength used to detect filipin. In order to visualize the filipin-stained cholesterol, cells were grown on glass coverslips in 35 mm plates and deprived of FBS overnight. The following day, they were treated with 100 μM berberine, and then fixed and stained with filipin. Images of the cells were captured with a fluorescence microscope and digital camera (Figure 3.6). Due to rapid photo-bleaching of filipin, accurate quantification of the fluorescent staining intensity by this method was not possible. Instead, cells were grown in a black-bottomed 96-well plate, stimulated and treated with berberine as described above, and then fixed and stained with filipin. Fluorescent intensity was measured in a plate reader, which ensured that all wells were measured rapidly and with equal exposure to the excitation light source. Fluorescent intensity in berberine-treated cells was observed to be 20% lower than in control cells (Figure 3.7).

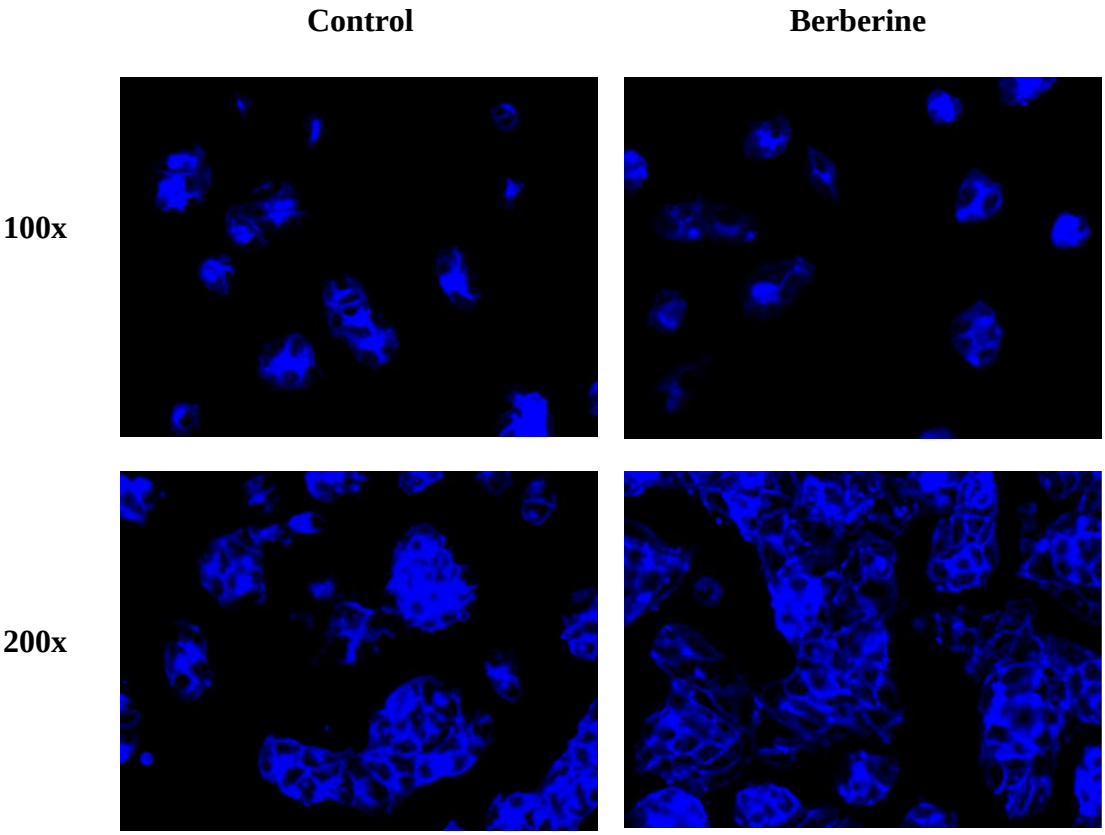

Figure 3.6 HepG2 Cells Stained with Cholesterol-binding Dye Filipin

HepG2 cells were seeded on sterile glass coverslips in 35 mm culture dishes and HMG-CoA reductase activity was stimulated by overnight serum deprivation. The cells were then treated with 100 μM berberine for 6 hrs and stained with filipin. The cells were immediately viewed with a fluorescence microscope and photographed to visualize the intracellular free cholesterol. Representative photographs are shown.

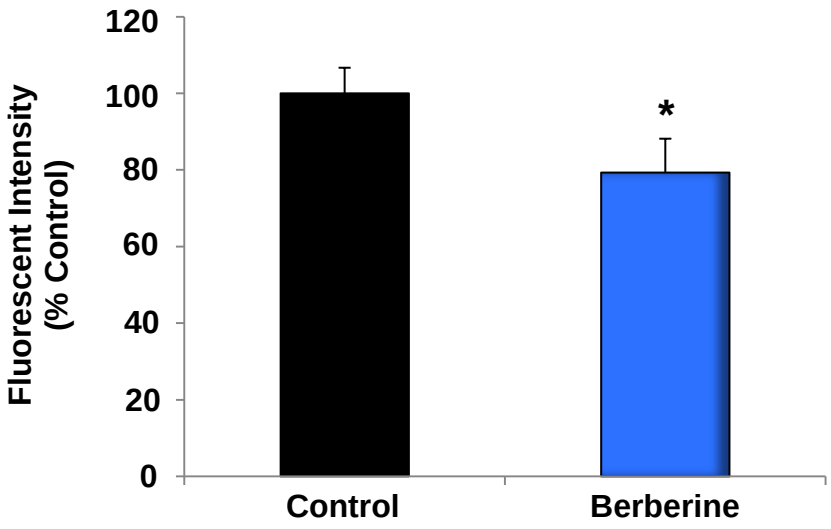

Figure 3.7 The Effect of Berberine on Intracellular Cholesterol Levels

HepG2 cells were cultured in a black-bottomed plate and HMG-CoA reductase activity was stimulated by overnight serum deprivation. The cells were then treated with berberine (100 μM for 6 hrs) and stained with the fluorescent cholesterol-binding dye filipin. Fluorescent intensity was measured in a plate reader.

*p<0.005 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

3.2 Investigation of the Underlying Mechanism of Berberine's Inhibitory Effect on HMG-CoA Reductase

The second objective of this study was to determine the mechanism by which berberine inhibited HMG-CoA reductase. HMG-CoA reductase is extensively regulated by transcriptional, translational and post-translational mechanisms (Nakanishi et al., 1988; Goldstein and Brown, 1990; DeBose-Boyd, 2008). We investigated the ability of berberine to inhibit HMG-CoA reductase by directly binding to the enzyme, to alter HMG-CoA reductase gene expression and to affect HMG-CoA reductase protein levels, in order to determine how berberine brings about a decrease in intracellular cholesterol.

3.2.1 Direct Binding of Inhibitor

The simplest mechanism for inhibition of an enzyme is the direct binding of the inhibitor to the enzyme in a way that blocks the catalytic site or changes the conformation of the enzyme so that it cannot efficiently catalyze the reaction. We performed an enzyme activity assay in HepG2 cell lysate to determine whether berberine might inhibit HMG-CoA reductase by binding to it directly (in a manner similar to the mechanism of statins). If berberine were acting in this way, we would expect that addition of berberine to HepG2 cell lysate would result in a measurable decrease in HMG-CoA reductase activity. However, there was no significant change in HMG-CoA reductase activity when berberine was added directly to the cell lysate (Figure 3.8).

3.2.2 Gene expression

Total RNA was isolated from cells stimulated overnight and treated with 100 μM berberine for 6 hrs. Real-time quantitative PCR analysis was run to determine whether berberine had an effect on the expression of HMG-CoA reductase in HepG2 cells.

Surprisingly, treatment with berberine increased gene expression of HMG-CoA reductase 2- to 3-fold in HepG2 cells (Figure 3.9).

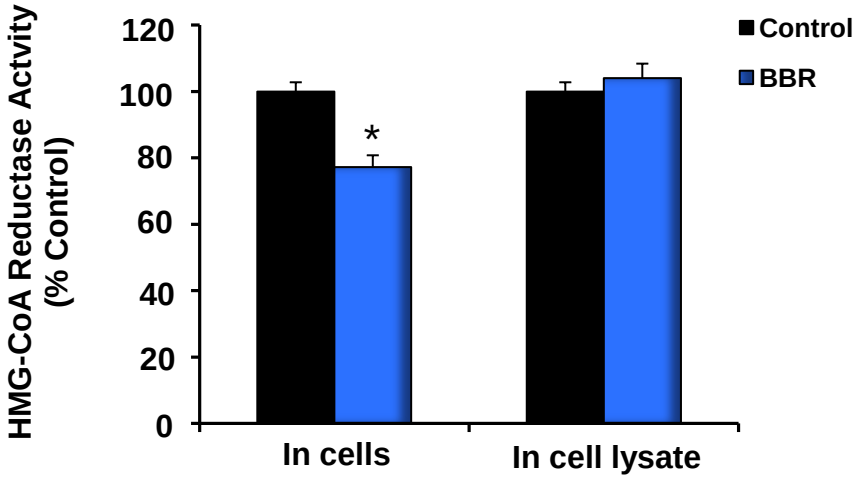

Figure 3.8 The Effect of Berberine on HMG-CoA Reductase Activity in HepG2 Cell Lysate.

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation, and the cells were collected and lysed. HepG2 cell lysate was incubated in the absence (control) or presence of berberine (BBR; 100 μM) for 30 min before assaying for enzyme activity. In contrast to the effect of berberine in living cells, berberine had no significant inhibitory effect on HMG-CoA reductase activity when added directly to cell lysate.

*p<0.001 compared with control values (expressed as 100%). Results were expressed as mean ± SEM.

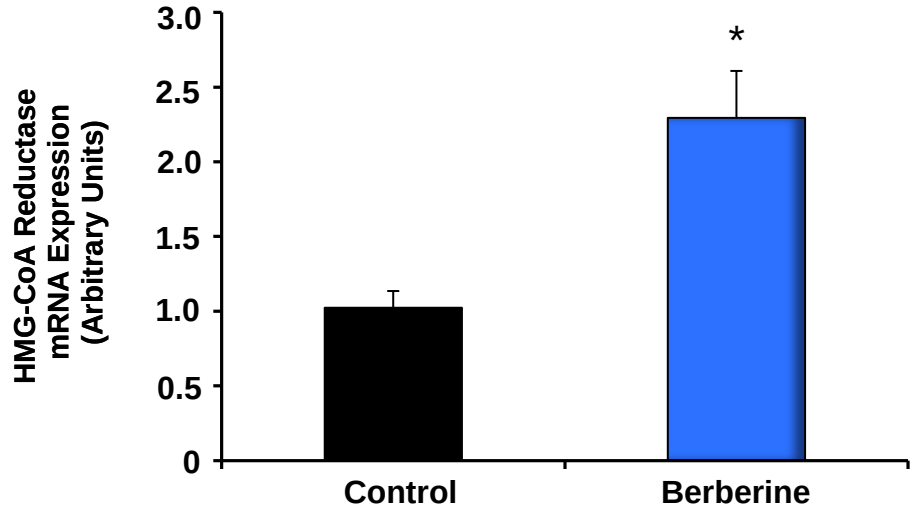

Figure 3.9 Effect of Berberine on HMG-CoA Reductase mRNA Expression

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation. The following day, HepG2 cells were treated with 100 μM berberine for 6 hrs and total RNA was isolated. HMG-CoA reductase mRNA expression was determined by quantitative real-time PCR.

*p<0.005 compared with control values (expressed as 1.0, arbitrary units). Results were expressed as mean ± SEM.

3.2.3 Protein expression

Initially, we measured total HMG-CoA reductase protein levels after berberine treatment and found no significant difference in cells that had been treated with berberine and control cells (Figure 3.10). However, upon examination of the literature, we discovered that berberine had been shown to activate AMPK, a cellular protein kinase that phosphorylates HMG-CoA reductase, inhibiting it. This finding encouraged us to measure the levels of phosphorylated HMG-CoA reductase (phospho-HMGR) after treatment with berberine. Phospho-HMG-CoA reductase was increased by approximately 33% in cells treated with 100 μM berberine for 6 hrs compared to control cells (Figure 3.10). Total HMG-CoA reductase levels did not change significantly after berberine treatment, indicating that the increase in phospho-HMG-CoA reductase was not the result of increased gene transcription. Next we measured the levels of total AMPK and phospho-AMPK (the activated form) after 30 min treatment with berberine (100 μM), and found a 2-fold increase in phospho-AMPK (Figure 3.11). Total AMPK protein levels did not change significantly (Figure 3.11).

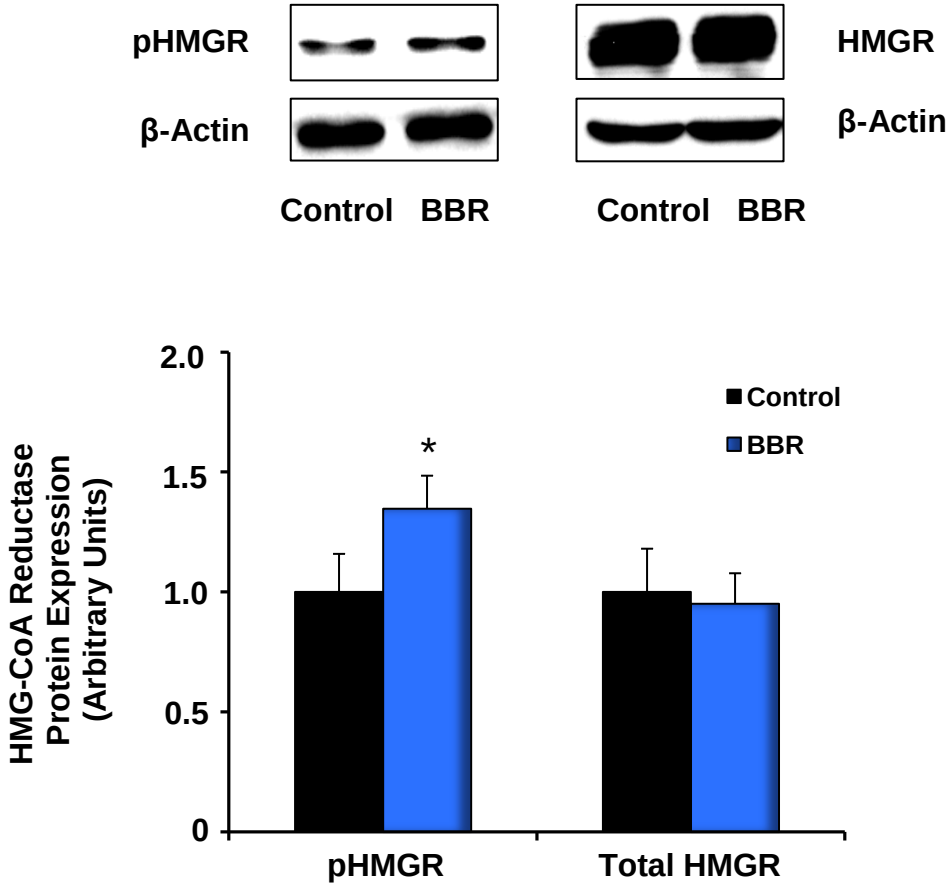

Figure 3.10 Effect of Berberine on HMG-CoA Reductase Protein Levels

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation. HepG2 cells were then treated with berberine (100 μM) for 6 hrs and the cellular protein fraction was purified. Protein levels of phospho-HMG-CoA reductase (pHMGR) and total HMG-CoA reductase were assessed by Western immunoblotting analysis.

*$p<0.05$ compared with control values (expressed as 1.0, arbitrary units). Results were expressed as mean ± SEM.

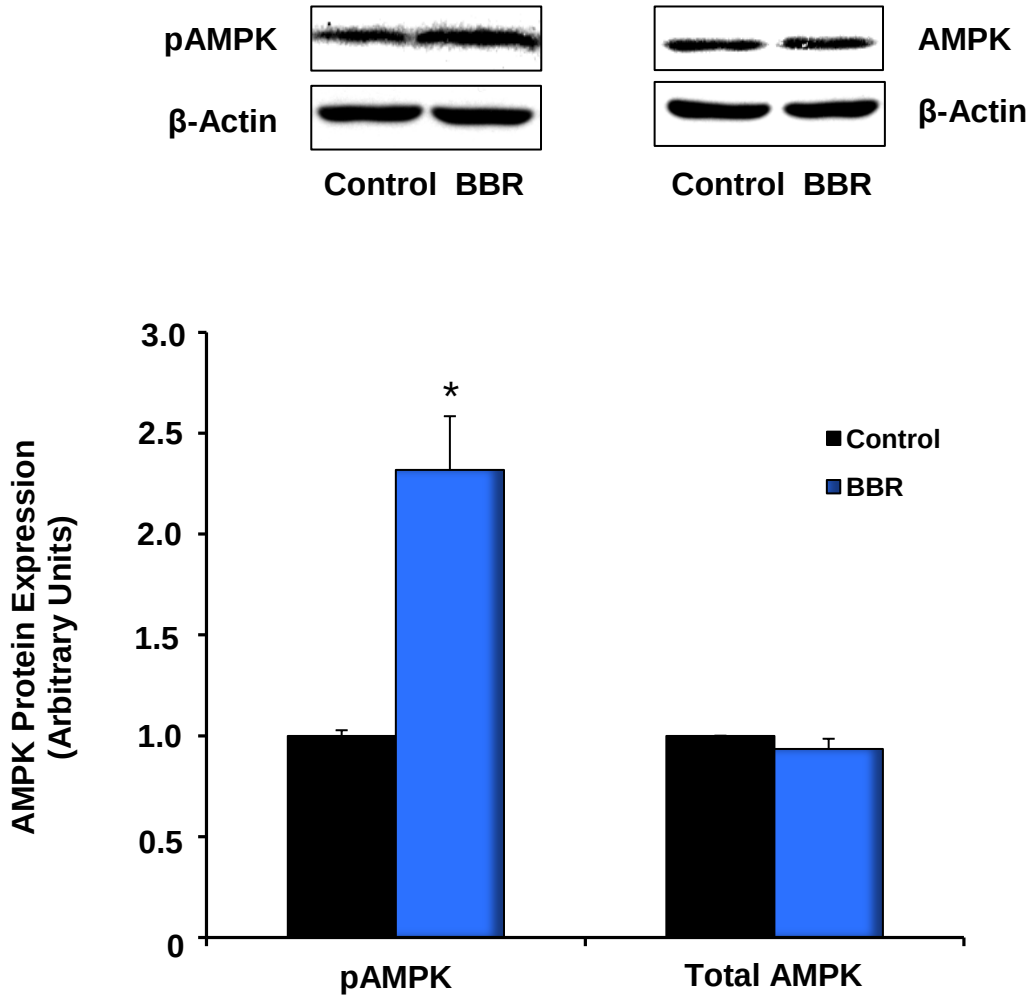

Figure 3.11 Effect of Berberine on AMPK Protein Levels

HMG-CoA reductase activity in HepG2 cells was stimulated by overnight serum-deprivation. HepG2 cells were then treated with berberine (BBR; 100 μM) for 30 min. The cellular protein fraction was purified, and the levels of phospho-AMPK (pAMPK) and total AMPK were assessed by Western immunoblotting analysis.

*$p<0.05$ compared with control values (expressed as 1.0, arbitrary units). Results were expressed as mean ± SEM.

Chapter 4

Discussion

4.1 The Role of Berberine in the Regulation of Lipid Metabolism

Berberine has previously been shown to play an important role in regulating lipid metabolism by two distinct mechanisms. First, berberine acts to increase the stability of LDL receptor mRNA (Kong et al., 2004). The consequent increase in LDL receptor in hepatocytes brings about improved clearance of LDL-C from the plasma. Additionally, berberine has been shown to regulate lipid metabolism through the cellular energy sensor AMPK. Berberine initiates a signalling cascade, causing the activation of AMPK by phosphorylation. AMPK in turn phosphorylates various downstream targets, affecting their activities (Brusq et al., 2006). One of these targets is the enzyme acetyl-CoA carboxylase (ACC), which is involved in triglyceride metabolism. Phosphorylation of ACC increases fatty acid oxidation and lowers the rate of triglyceride synthesis. These two findings have been the key mechanistic pathways in explaining berberine's beneficial effects on the lipid profile.

In the present study, we demonstrated that berberine exerts its lipid-lowering effect in an additional and complementary way. Berberine was identified as a regulator of HMG-CoA reductase through the activation of AMPK and the subsequent deactivation of HMG-CoA reductase by AMPK (Figure 4.1). It has previously been shown that berberine activates AMPK and that AMPK has multiple downstream effects on target enzymes and pathways, but the relationship between berberine and HMG-CoA reductase had not been previously established.

We initially assessed the effect of berberine on HMG-CoA reductase activity in serum-deprived HepG2 cells. Overnight serum deprivation resulted in a large increase in HMG-CoA reductase activity, similar to the hepatic condition of individuals suffering from

familial hypercholesterolemia. Berberine was effective in significantly reducing HMG-CoA reductase activity in the stimulated cells, although not to baseline levels. Additionally, we noted a decrease in HMG-CoA reductase activity when we added berberine to HepG2 cells with baseline HMG-CoA reductase activity. Although this phenomenon was not further explored in this study, the finding that berberine could inhibit baseline HMG-CoA reductase activity suggests that berberine may be useful in lipid-lowering not only in those patients with elevated HMG-CoA reductase activities, but in other hypercholesterolemic patients as well.

In order to determine whether berberine affected HMG-CoA reductase gene expression, we conducted a real-time PCR analysis on RNAs isolated from serum-deprived cells that had been treated with berberine. To our surprise, we found a significant increase in HMG-CoA reductase mRNA expression in cells that had been treated with berberine. It is likely that this increase occurred as a result of suppression of cholesterol synthesis in the cells, similar to the way statins inhibit HMG-CoA reductase and thereby activate the negative feedback loop that results in increased expression of HMG-CoA reductase and LDL receptor. We suspect that HMG-CoA reductase mRNA expression is activated by the SREBP pathway in response to lowered cholesterol levels in the cells.

In our study, berberine was shown to decrease the activity of HMG-CoA reductase through the activation of AMPK. We showed by Western immunoblotting analysis that treatment of HepG2 cells with berberine caused a significant increase in phospho-AMPK (activated) within 30 minutes, whereas total AMPK protein levels did not change. After 6 hrs of berberine treatment, a consequent increase in phospho-HMG-CoA reductase (inactivated) was observed, while total HMG-CoA reductase protein levels stayed stable.

The reason for the increase in HMG-CoA reductase mRNA with no change in protein levels remains something of an enigma. A possible explanation for this discrepancy is berberine's proven ability to regulate mRNA stability. Experiments in Kong et al.'s study (Kong et al., 2004) demonstrated that there was no change in HMG-CoA reductase mRNA stability upon treatment of HepG2 cells with berberine. However, a presently unexplored means by which the increase in mRNA expression could have occurred are a change in the rate of mRNA degradation. This represents a possible mechanism by which the effects of berberine in the cell could be further expressed. Berberine has been shown to improve LDLR mRNA stability by regulating several types of mRNA binding proteins (Li et al., 2009). However, the details of how the process of mRNA stabilization occurs or which other cellular factors are involved are not known. Another recent study links berberine to mRNA decay in a macrophage model of the inflammatory response (Zha et al., 2010). Berberine's ability to influence mRNA stability is a likely mechanism for the large increase in mRNA expression observed in this study.

Downstream of berberine's effects on mRNA and protein expression, we observed a decline in hepatocellular cholesterol content as the end result of inhibiting HMG-CoA reductase. We demonstrated this by staining the free cholesterol in the cells with the fluorescent dye filipin and measuring fluorescent intensity. Our results suggest that the decrease in LDL-C seen in animals and humans treated with berberine may be the result of berberine's effect on HMG-CoA reductase phosphorylation state, LDLR mRNA and triglyceride synthesis.

Our results support berberine's potential as a marketable hypocholesterolemic drug, because it demonstrates low toxicity and was generally very well tolerated by human

participants in clinical trials (Kong et al., 2004; Yin et al., 2008b; Zhang et al., 2008). Berberine acts via several different mechanisms to bring about its effects on lipid metabolism. It has been suggested that since berberine and statins achieve the same result, an up-regulation of LDLR, berberine would be unlikely to replace statins as a hypocholesterolemic agent (Doggrell, 2005). We would argue that, because statin treatment is sometimes discontinued due to side effects such as muscle pain, berberine provides an attractive alternative, having already demonstrated its low toxicity in human trials, particularly in cases where muscle-related side effects occur. Furthermore, animal experiments and clinical trials combining berberine and statins to lower lipids have demonstrated that significantly greater lipid-lowering effects can be achieved using lower doses of berberine and simvastatin in combination than with either monotherapy (Kong et al., 2008).

It is recognized that in comparison to statins, the overall effects of berberine in lipid lowering are not dramatic. In the HepG2 cell model in this study, berberine lowered cholesterol by 25-30%, whereas statins have been shown to reduce cholesterol levels in HepG2 cells by up to 65% (Scharnagl et al., 2001). Kong et al. (Kong et al., 2004) showed that berberine lowered cholesterol in hypercholesterolemic hamsters (up to 42%) and in humans (25%). On the other hand, statins have been shown to lower serum LDL-C by as much as 60% in humans (LaRosa et al., 1999; Yeung and Tsao, 2002). While there is no doubt that statins are powerful lipid-lowering drugs, the fact remains that they are not a suitable option for all patients due to their in some cases intolerable side effects. Additionally, the dose dependent lipid-lowering effect of berberine indicates that there is potential for greater decreases in lipids with this natural health product. While the present

study is limited in scope due to the use of a cell model system, other studies in animal models and human trials have demonstrated the benefits of berberine in lipid metabolism regulation. Further investigation is warranted to determine whether berberine could be marketed as a lipid-lowering therapy and at what dosage it may confer its greatest benefit.

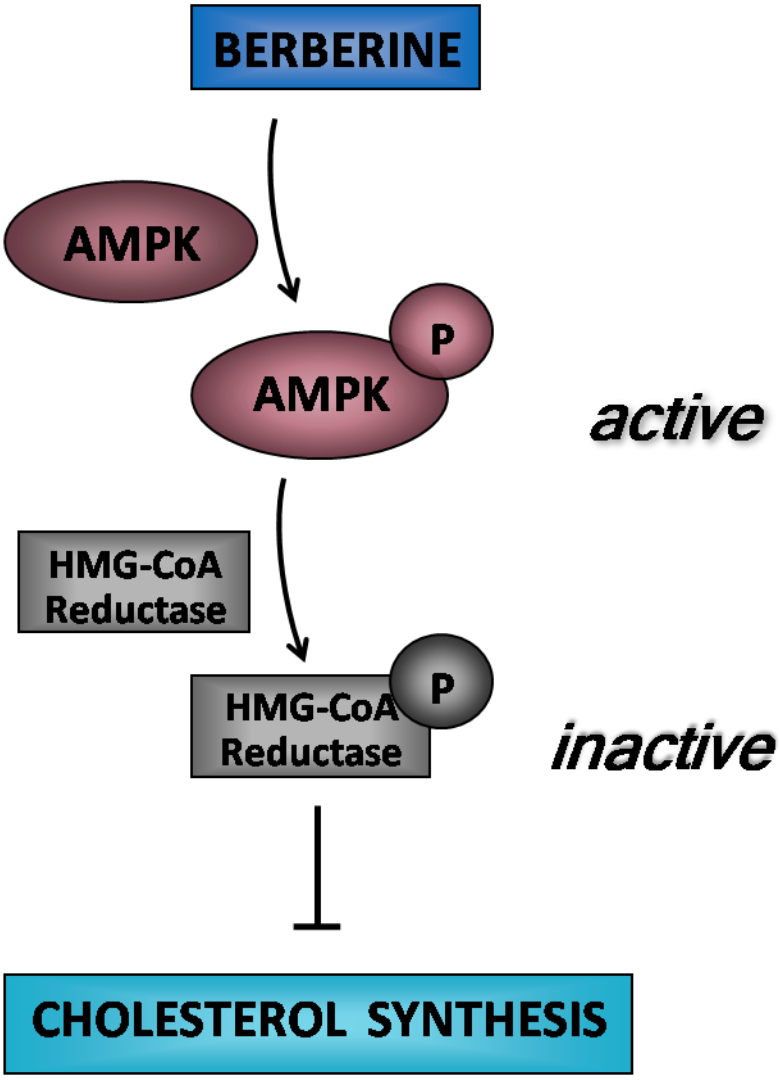

Figure 4.1 The Berberine-AMPK-HMG-CoA Reductase Pathway

The results of this study demonstrate that berberine activates AMPK by causing it to be phosphorylated. One of the downstream effects of AMPK activation is phosphorylation of HMG-CoA reductase, which inactivates the reductase and blocks cholesterol synthesis.

4.2 The Role of Berberine in Energy Metabolism and Disease

The pathway outlined by the present study (Figure 4.1) confirms AMPK's role as a central energy regulator in mammalian cells. AMPK is a serine/threonine protein kinase and a master regulator of the metabolic pathways involved in ATP production, consumption and maintenance of homeostasis, as well as playing a role in cell growth, proliferation and apoptosis (Hue et al., 2003; Young et al., 2005; Hardie, 2007). AMPK is a heterotrimeric complex consisting of the catalytic α-subunit and the regulatory β- and γ-subunits. Several isoforms of each subunit are expressed in humans and rodents in different tissues (Kahn et al., 2005). The α-subunit contains a conserved threonine residue (Thr-172), phosphorylation of which is essential for activation. The primary kinase that activates AMPK has been identified as LKB1, a tumour suppressor protein mutated in Peutz-Jeghers syndrome, which operates with two accessory subunits, STRAD and MO25. It has been suggested that the major role AMPK plays in cell cycle and growth makes it likely that this kinase mediates many of the tumour suppressor effects of LKB1 (Hardie, 2008). Numerous lines of evidence also indicate a role for berberine as an anti-cancer agent (Choi et al., 2009; Hur et al., 2009; Tsang et al., 2009), and it is possible that berberine exerts these effects as an upstream activator of AMPK. AMPK acts through multiple mechanisms to arrest cell proliferation and to inhibit protein synthesis (Igata et al., 2005). By these same mechanisms, AMPK activation may be beneficial in preventing the progression of malignant tumours or atherosclerotic plaque development (Motoshima et al., 2006; Zang et al., 2006) and may be a valuable target for anti-cancer therapy. However, a recent study has demonstrated that berberine activated AMPK independently of LKB1 in $LKB1^{-/-}$ cells (Turner et al., 2008), highlighting the

importance of gaining a more thorough understanding of berberine signalling pathways. The majority of studies dealing with berberine and AMPK are focused on pathways in muscle or adipose tissue. Two recent studies have identified mechanisms by which berberine activated AMPK. In one case, berberine was shown to inhibit respiratory complex I in the mitochondria of mouse embroyonic fibroblasts (Turner et al., 2008). The role of the respiratory chain in the mitochondrial membrane is ATP production, and the inhibition of one of the chain's components causes a drop in cellular ATP, thus activating AMPK. Respiratory complex I is also a target for anti-diabetic drug metformin, which is known to improve insulin sensitivity by suppressing glucose production (Kim et al., 2008b). It has been suggested that berberine's anti-diabetic properties are a result of its ability to hinder respiratory complex I (Turner et al., 2008). Another study demonstrated berberine's ability to induce glycolysis (Yin et al., 2008a). The authors observed that oxygen consumption was reduced in adipocytes and myotubes by berberine, consistent with the above-mentioned study. The decline in aerobic respiration was associated with an increase in anaerobic respiration (glycolysis), a process in which glucose is utilized to produce ATP, albeit in a less efficient manner than aerobic respiration. Since more glucose is required to produce ATP by anaerobic respiration, berberine was shown to promote glucose utilization and uptake by this mechanism. AMPK activation occurred in adipocytes and myotubes as a result of persistent mitochondrial inhibition by berberine (Yin et al., 2008a).

The critical role of LKB1 and AMPK in regulation of glucose metabolism in the liver has recently been demonstrated. Mice lacking the LKB1 gene have low hepatic phospho-AMPK levels and low AMPK activity (Shaw et al., 2005). These animals have marked

hyperglycemia and glucose intolerance, a pre-diabetic state of glucose metabolism dysregulation associated with insulin resistance and increased risk of cardiovascular pathology. Berberine, as an activator of hepatic AMPK, has the potential to alleviate these negative effects.

The literature presents AMPK as an important homeostatic mediator under conditions of stress, including starvation. Starvation (glucose deprivation) leads to AMPK activation and results in the suppression of glucose-consuming pathways. Therefore, one would perhaps expect that in the model utilized in the present study, in which HepG2 cells are serum-deprived, AMPK would be activated before the cells were ever treated with berberine. Under such conditions, one would anticipate that the inactivated phosphorylated form of HMG-CoA reductase would predominate. This, however, is not the case, since HMG-CoA reductase activity increases in a time-dependent manner during serum-deprivation, as shown in Figure 2.1. The scope of this project does not allow for a thorough investigation of all the mechanisms and signalling pathways involved in HMG-CoA reductase activity up-regulation through serum deprivation and AMPK activation. Nevertheless, it is clear that glucose deprivation and serum deprivation create distinct metabolic environments in the cell. This paradox emphasizes the complexity of the relationships between lipid and glucose metabolism in hepatic cells. The relationship between berberine and HMG-CoA reductase demonstrated in the present study suggests a role for berberine in energy metabolism. Cholesterol synthesis is an energetically expensive process, requiring 18 molecules of ATP per molecule of cholesterol, as well as molecular oxygen and reducing power in the form of NADPH. As cellular levels of ATP are depleted, AMPK senses the increasing AMP levels through

AMP-binding sites in its γ-subunit (Scott et al., 2004) and suppresses cholesterol synthesis by phosphorylating HMG-CoA reductase. This pathway is particularly important in skeletal muscle, where frequent contractions can rapidly deplete the cells' supply of ATP. Berberine may play a role in restoring energy balance in skeletal muscle. In summary, berberine appears to have many and varied roles in energy metabolism and metabolic disorders. Further studies investigating berberine's activities will provide insight into the health potential of this natural product.

Chapter 5

Summary and Conclusions

Summary

The present study reveals a novel mechanism for berberine's role in regulating cholesterol metabolism. Our results demonstrate that berberine activates a signalling pathway involving cellular energy sensor AMPK to bring about post-translational modification of HMG-CoA reductase and thereby decrease the activity of this enzyme. The findings presented here contribute to our current understanding of berberine's actions in lipid metabolism, summarized in Figure 5.1. Furthermore, this study supports the outcomes of clinical trials involving berberine as a regulator of lipid metabolism.

Conclusions

Berberine has the potential for becoming a new lipid-lowering agent. Berberine has been shown to act via multiple mechanisms to decrease cholesterol and triglycerides, all of which are distinct from those employed by current hypolipidemic therapies on the market. For many patients, taking a natural health product is preferable to a synthetic drug, such as when intolerance to high dosages of a drug makes reaching lipid-lowering goals improbable. Studies suggest berberine may also have added benefits in combination therapy with statins, and further investigation into this area is ongoing.

In addition to its effects on lipid metabolism, many preliminary studies suggest a role for berberine in widespread areas of disease, including mental health disorders, cardiovascular disease and cancer. Future research may demonstrate further benefits of the natural health product berberine.

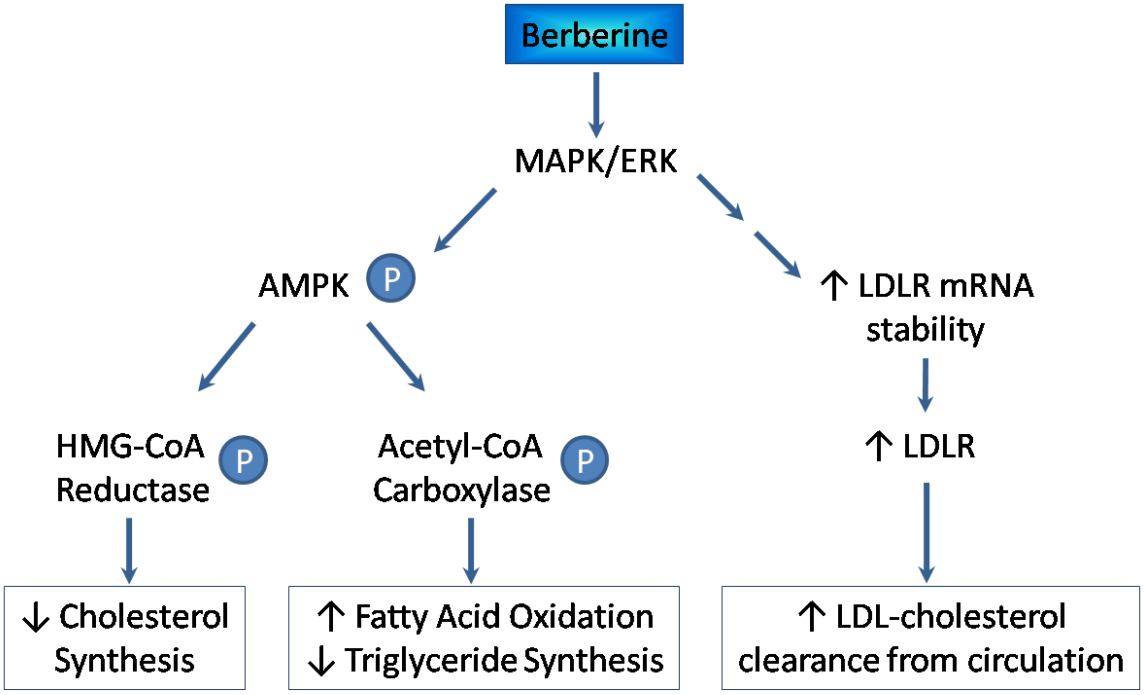

Figure 5.1 Summary of Berberine's Role in Regulating Lipid Metabolism

Berberine has previously been shown to regulate lipid metabolism via two distinct pathways. By increasing LDLR mRNA stability, berberine improves LDL-C clearance from the circulation. Berberine activates AMPK, which phosphorylates downstream targets, such as acetyl-CoA carboxylase, thereby promoting fatty acid oxidation. The present study demonstrates that berberine also inhibits cholesterol synthesis through the post-translational modification of HMG-CoA reductase by AMPK. The phosphorylated form of HMG-CoA reductase is inactive. This finding contributes to our overall understanding of berberine's role in the regulation of lipid metabolism.

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Appendix I

Reagents/Chemicals	*Company*
Acetone	EMD Chemicals
Acrylamide/bis 30% solution 37.5:1 (2.6% C)	Bio-Rad
Anti-AMPK antibody	Cell Signalling Technology
Anti-HMG-CoA Reductase antibody	Upstate
Anti-phospho-AMPK antibody	Cell Signalling Technology
Anti-phospho-HMG-CoA Reductase antibody	Upstate
Anti-rabbit IgG, HRP-linked antibody	Zymed
Berberine chloride, BBR	Sigma-Aldrich
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad
Bovine serum albumin, BSA	EMD Chemicals
Bromophenol blue	Sigma-Aldrich
Calcium chloride ($CaCl_2$-H_2O)	Fisher Scientific
Chloroform ($CHCl_3$)	Fisher Scientific
Diethyl pyrocarbonate, DEPC	Sigma-Aldrich
Dithiothreitol, DTT	Sigma-Aldrich
Dimethyl sufoxide, DMSO	Fisher Scientific
Disodium pyrophosphate	Sigma-Aldrich
dNTPs	Invitrogen
Dulbecco's modified Eagle's medium, DMEM	Hyclone
Ethylene glycol tetraacetic acid, EGTA	Sigma-Aldrich

Ethylenediaminetetraacetic acid, EDTA	Sigma-Aldrich
Foetal bovine serum, FBS	Hyclone
Filipin complex from Streptomyces filipinensis	Sigma-Aldrich
First strand buffer	Invitrogen
Formaldehyde	Sigma-Aldrich
Glucose, D-	Sigma-Aldrich
Glucose-6-Phosphate, G6P	Sigma-Aldrich
Glucose-6-Phosphate dehydrogenase, G6PDH	Sigma-Aldrich
β-glycerophosphate	Sigma-Aldrich
Glycerol	Fisher Scientific
Hank's balanced salt solution, HBSS	Hyclone
Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Fisher Scientific
HMG-CoA	Sigma-Aldrich
[3-^{14}C]HMG-CoA	PerkinElmer Life Sciences
Hydrochloric Acid, HCl	Fisher Scientific
Hydrogen peroxide, 30%	Fisher Scientific
Isopropanol	Fisher Scientific
iQ-SYBR green supermix reagent	Bio-Rad
Leupeptin	Sigma-Aldrich
Lipid calibrator	Wako USA
Mercaptoethanol, β-	Sigma-Aldrich
Mevalonolactone, ML	Sigma-Aldrich
M-MLV-Reverse transcriptase (200U/ml)	Invitrogen

3-(3,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT	Bio Basic Inc.
Neutral buffered formalin (10%)	Sigma-Aldrich
Nicotinamide adenine dinucleotide phosphate sodium salt (reduced), NADPH	Sigma-Aldrich
Oligo$(dT)_{12\text{-}18}$ primer, 0.5 ug/ul	Invitrogen
PCR buffer 10x	New England Biolab
Phenolmethanesulfonyl fluoride, PMSF	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Potassium phosphate, dibasic	Sigma-Aldrich
Protein assay reagent	Bio-Rad
RNase inhibitor	Promega
Sodium chloride	VWR
Sodium dodecyl sulfate	Fisher Scientific
Sodium orthovanadate	Sigma-Aldrich
Sucrose	Fisher Scientific
Toluene	Sigma-Aldrich
Tris	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
TriZol Reagent	Invitrogen
Trypsin-EDTA 10x	Gibco
Tween	Fisher Scientific

Appendix II

Buffers	***Components***
Cell Lysis Buffer	50mM Tris-HCl, 150mM NaCl, pH 7.4
Phosphate Buffer 1	50 mM K_2HPO_4, 5 mM DTT, 1 mM EDTA, pH 7.4
Phosphate Buffer 2	100 mM K_2HPO_4, 10 mM DTT, 2 mM EDTA, pH 7.4
Phosphate buffered solution, PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 KH_2PO_4, pH 7.4
Protein Lysis Buffer	20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2.1 μM leupeptin, 1 mM PMSF, 1% Triton-X 100
Sodium Buffer (Filipin staining)	150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 20 mM Hepes, 2g/L glucose, pH 7.4

Appendix III

Equipment	*Model*	*Company*
Centrifuge	5804 R	Eppendorf
Incubator	Forma Direct Heat CO_2	Thermo Electron Corporation
Waterbath	Isotemp 205	Fisher Scientific
Scintillation Counter	LS 6500	Beckman Coulter
Spectrophotometer	DU 800	Beckman Coulter
Microplate Reader	SpectraMax M5	Molecular Devices
PCR Detection System	iQ5 real-time PCR	Bio-Rad
Gel Doc System	Gel Doc 1000	Bio-Rad
Fluorescence Microscope	Axioskop2 MOT	Zeiss