GREEN TEA AND ITS CATECHINS MODULATE CHOLESTEROL METABOLISM IN CULTURED HUMAN LIVER (HEPG2) CELLS AND THE HYPERCHOLESTEROLAEMIC RABBIT.

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CHAPTER 3

FRESHLY BREWED GREEN TEA MODULATES CHOLESTEROL METABOLISM IN CULTURED HUMAN LIVER (HEPG2) CELLS.

ABSTRACT

Hypercholesterolaemia is one of the main risk factors in the development of heart disease. Green tea and its antioxidant constituents, the catechins, have been found to be hypocholesterolaemic in both epidemiological and animal intervention studies. Previous studies in our laboratory have found that freshly brewed green tea and its most abundant catechin constituent epigallocatechin gallate (EGCG), increased the low-density lipoprotein (LDL) receptor of HepG2 cells. As an increase in the low-density lipoprotein receptor is one mechanism by which plasma cholesterol levels can be lowered, this could explain the hypocholesterolaemic effects that have been found with green tea and its catechins in the epidemiological and animal intervention studies.

The main objectives of the present studies were to investigate the mechanism by which green tea and EGCG increase the LDL receptor in HepG2 cells. The LDL receptor can be regulated through changes in cellular cholesterol content, which modulates the level of the mature active form of sterol regulatory element binding proteins (SREBPs), transcription factors for the LDL receptor. These parameters were therefore investigated. Furthermore, we wanted to determine if a crude catechin extract from green tea could lower plasma cholesterol levels in the hypercholesterolaemic rabbit and ascertain if this effect was due to an increase in the LDL receptor.

Green tea and EGCG significantly decreased cellular total cholesterol (~30%) at all treatment concentrations ($p<0.05$). There are three main mechanisms by which this could occur in liver cells: 1) an increase in the conversion of cholesterol into bile acids 2) an inhibition in cholesterol synthesis or 3) an increase in the efflux of cholesterol from the cells to the media. Chenodeoxycholic acid, the main bile acid produced by HepG2 cells, was extracted from the cell media and measured using gas chromatography (GC). No changes were noted in its production after treatment with green tea or EGCG. The reduction in cellular total cholesterol concentrations was therefore not likely to be due to an increase in the conversion of cholesterol to bile acids.

Incubation with green tea and EGCG produced a bi-phasic "down then up" effect on cholesterol synthesis as measured using the cellular concentration of lathosterol relative to cell protein. The significant decrease (-33%) in cholesterol synthesis in the lowest dose treatment group (50 μM) could explain the decrease in cellular total cholesterol in those cells. In the highest dose treatment group (200 μM) however, there was an increase in cholesterol synthesis (+40%), which did not support the decrease in cellular total cholesterol. Further studies revealed that both green tea and EGCG, in the highest dose treatment group only, increased the concentration of cholesterol in the media (+25%). This suggested that the extra cholesterol produced by the increase in cholesterol synthesis, was not remaining in the cells but was secreted into the media. The decrease in the cell cholesterol by green tea and EGCG therefore appeared to be due to a decrease in cholesterol synthesis at the lowest dose but due to an increase in the secretion of cholesterol from the cells at the highest dose.

The decrease in cellular cholesterol is consistent with the LDL receptor being upregulated via the SREBP transcription system. Measurement of SREBP-1c, using a specific polyclonal antibody and western blotting, revealed that incubation of HepG2 cells with freshly brewed green tea and EGCG increased the mature active form of SREBP-1c by 65% and 56% over control levels respectively. This increase in the mature active form of SREBP-1c is therefore consistent with the increase in the LDL receptor seen with green tea and EGCG.

To determine if the effects of green tea and EGCG on HepG2 cell cholesterol metabolism also occurred *in vivo*, 24 New Zealand white rabbits were initially made hypercholesterolaemic by feeding them 0.25% (w/w) cholesterol mixed in with their normal rabbit chow for a period of 2 weeks. The rabbits were then randomised into four different treatment groups based on body weight and plasma cholesterol levels. The four treatment groups were then fed the 0.25% cholesterol diet supplemented with 0, 0.5, 1 or 2% (w/w) of a crude catechin extract from green tea. At the end of the treatment period the rabbits were bled via cardiac puncture until euthanasia and their livers and aortas were excised.

The administration of the crude catechin extract (2% w/w) to cholesterol-fed rabbits produced reductions in plasma cholesterol (-57%) and cholesterol in the VLDL + IDL (-80%) and the LDL (-77%) fractions compared to the controls. There was a significant inverse linear trend between plasma, VLDL + IDL and LDL cholesterol and the dose of the crude catechin extract ($p<0.05$). Reductions in total and unesterified cholesterol for the liver homogenate (25% and 15%) and the liver membrane (22% and 21%) fraction were also found. There were significant inverse linear trends between total and unesterified cholesterol in both liver preparations and the dose of the crude catechin extract ($p<0.05$).

There also was a significant inverse linear trend ($p<0.05$) between cholesterol in the thoracic aorta and the dose of the crude catechin extract (–22%). Fatty streak formation was assessed by lipophilic staining using oil red O and quantified by image analysis, but the percentage lipophilic stain in the aortic arches was not different after consumption of the crude catechin extract compared to the control diet.

Cholesterol synthesis, as measured by the plasma ratio of lathosterol to cholesterol, was significantly reduced in the 1% and 2% (w/w) treatment groups (-60%) compared to the control ($p<0.05$). This reduction in cholesterol synthesis is consistent with the various reductions observed in plasma, aorta and liver cholesterol with the administration of the crude catechin extract. Furthermore, cholesterol synthesis was significantly correlated to plasma, VLDL + IDL, LDL and aortic cholesterol ($r= 0.57, 0.56$ and 0.50 respectively).

An increase was noted in LDL receptor binding activity (+80%) in the 2% (w/w) treatment group compared to the control, measured by the calcium dependant binding of colloidal gold-LDL to solubilised liver membranes. There was also an increase in the relative amounts of LDL receptor protein (+70%) in the 2% (w/w) treatment group compared to the control, measured using a polyclonal antibody and western blotting. Significant positive linear trends between LDL receptor binding activity and LDL receptor protein and the dose of the crude catechin extract were observed ($p<0.05$). This increase in the LDL receptor

provides another mechanism to explain the reduction in plasma lipids that occurred with the administration of the crude catechin extract. It appears however that the reduction in cholesterol synthesis may be the main driving mechanism by which the crude catechin extract produces its cholesterol lowering effects as it is more strongly correlated with plasma lipids than the LDL receptor ($r = 0.37$ with total cholesterol).

In summary, the *in vitro* studies suggest that green tea and EGCG increase the LDL receptor by decreasing the cell cholesterol concentration and increasing the mature active form of SREBP-1c. The dietary intervention study revealed that the administration of a crude catechin extract to rabbits lowered plasma and LDL cholesterol. The mechanism by which the green tea extract lowered cholesterol in the rabbit appeared to be by reducing cholesterol synthesis and increasing the LDL receptor. This study provides evidence that green tea and its catechins exhibit hypocholesterolaemic properties and may therefore provide protection against heart disease.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Signed Date

.....20/12/00.....

Christina A Bursill

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ABBREVIATIONS

ACAT	acyl : cholesterol acyltransferase
ApoB	apolipoprotein B-100
CHD	coronary heart disease
DMEM	dulbecco's modified eagles media
EDTA	ethylenediaminetetra-acetic acid disodium salt
EGCG	(-) epigallocatechin gallate
FCS	fetal calf serum
HDL	high-density lipoprotein
HMGCoA reductase	β-hydroxy-β-methylglutaryl-coenzyme A reductase
IDL	intermediate-density lipoprotein
LDL	low-density lipoprotein
LPD-FCS	lipoprotein deficient-fetal calf serum
N-ALLN	N-acetyl-leucine-leucine-norleucinal
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
S1P	site-1 protease
S2P	site-2 protease
SCAP	SREBP cleavage-activating protein
SREBP	sterol regulatory element binding protein
VLDL	very low-density lipoprotein

PUBLICATIONS ARISING FROM THIS THESIS

Full Publications

Sebely Pal, Christina Bursill, , Cynthia D. K. Bottema, Paul D. Roach. 1999. Regualtion of the Low-Density Lipoprotein Receptor by Antioxidants *In* Antioxidants in Human Health and Disease. T. K. Basu, N. J. Temple and M. L. Garg, editors. CABI, Wallingford, U.K. Chapter 5 p55-69.

Christina Bursill, Paul D. Roach, Cynthia D. K. Bottema and Sebely Pal. Green tea upregulates the Low Density Lipoprotein Receptor of Human Liver Cells. *Atherosclerosis* (Submitted) 2000.

Christina Bursill, Mavis Abbey and Paul D Roach. A Green Tea Catechin Extract lowers plasma cholesterol in the Cholesterol-fed Rabbit. *J of Nutrition* (Submitted) 2000.

Christina Bursill, Mavis Abbey and Paul D Roach. Epigallocatechin gallate upregulates the low-density lipoprotein receptor in human liver cells. *J Lipid Res.* (Submitted) 2000.

Abstracts

CA Bursill and PD Roach. Regulation of the low density lipoprotein receptor by green tea and epigallocatechin gallate. *Proceedings of the Australian Atherosclerosis Society, 1998.*

CA Bursill and PD Roach. Green tea and epigallocatechin gallate decrease cholesterol concentrations and inhibit cholesterol synthesis in HepG2 cells. *Proceedings of the Nutrition Society of Australia, 1998;22:281.*

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CA Bursill, M Abbey and PD Roach. Green tea catechin extract beneficially modifies cholesterol metabolism in the hypercholesterolaemic rabbit. *International Atherosclerosis Symposium, 2000, p 109.*

CA Bursill, PD Roach. Green tea and Epigallocatechin gallate modulate cholesterol metabolism in cultured human liver cells. *International Atherosclerosis Symposium, 2000, p 110* .

Chapter 1

Introduction

INTRODUCTION

1.1 Cholesterol and Heart Disease

Cholesterol is a sterol that occurs in man in a free (unesterified) and esterified form. It is essential in the body as it is a component of all cell membranes and is used in the production of steroid hormones and bile acids. Despite this, however, elevated levels of cholesterol in the blood are a major risk factor for coronary heart disease (CHD), the leading cause of mortality in Western society. Evidence for this has accumulated from many avenues of investigation including epidemiological studies, animal experiments and genetic models.

Epidemiological studies suggest that the incidence of CHD is relatively constant for blood cholesterol levels up to 5.2 mmol/L but above this threshold range the risk for CHD increases as cholesterol concentrations increase (Kannel *et al.*, 1971, Grundy, 1997, Rywik *et al.*, 1999). The National Heart Foundation therefore recommends that plasma cholesterol levels should not exceed 5.2 mmol/L. This link between hypercholesterolaemia and CHD has provided much of the impetus behind the research into cholesterol homeostasis and ways in which dietary and pharmacological intervention may act to lower plasma cholesterol and the incidence of CHD.

There are other factors that can play a role in the development of CHD; these include: high blood pressure, smoking, obesity, diabetes mellitus, dietary factors, age, gender, family history and physical inactivity (Kannel *et al.*, 1964, Durrington and Sniderman

2000). In more recent times, it has been found that elevated levels of homocystein are also positively related to CHD (Chen *et al.* 2000).

1.2 Cholesterol

1.2.1 Cholesterol Synthesis

The body can acquire cholesterol via two sources. It can be either absorbed from the diet or synthesised *de novo*. Cholesterol synthesis can play an important role during active growth or when dietary intake is limited (e.g. famine), however it is not an essential process in well-nourished people without evidence of ill health.

Almost all tissues and organs can synthesise cholesterol but under normal circumstances, newly synthesised cholesterol in the body originates from the small intestine and the liver. The liver is the main organ for cholesterol synthesis and is responsible for at least 50% of total body synthesis (Rudney and Sankhavaram, 1993). Cholesterol synthesis is an extremely complex process that starts with acetyl-CoA (Figure 1.1). Early on in this process an important physiological regulatory event occurs when 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) is converted to mevalonic acid (Durrington and Sniderman, 2000). The rate-limiting enzyme involved in this reaction is HMG-CoA reductase; it can be regulated by a variety of physiological factors at the level of gene transcription, mainly via the sterol regulatory elements (SRE) in the promoter region of the genes involved. Unlike other parameters of cholesterol metabolism (e.g. the low-density lipoprotein receptor), it can also be regulated at the translation and enzymatic level (Rudling, 1992). The most important effector in these regulatory processes is intracellular cholesterol concentrations, i.e.

the end product of the biosynthetic pathway. Therefore, any factor that affects the amount of cholesterol in the cell will ultimately alter the rate of cholesterol synthesis via this feedback mechanism (Havel, 1988).

The rate of cholesterol produced by the liver is also highly responsive to cholesterol absorption from the diet, for example, when cholesterol absorption is inhibited cholesterol synthesis is increased (Dory *et al.*, 1990) and vice versa. It will also be downregulated if there is an increased entry of cholesterol into the hepatic cells (probably due to an increase in dietary cholesterol). Drug therapy can alter cholesterol synthesis and is used in people with "high risk" levels of plasma cholesterol to lower its concentration. The HMG-CoA reductase enzyme is the main site of action for drug therapy (e.g. the statins) and inhibition of this enzyme has been shown to lower plasma cholesterol levels by 15-30% (Reihner *et al.*, 1990, Parker *et al.*, 1990, Endo 1992). This dramatic reduction in cholesterol has been shown to consequently reduce CHD deaths and myocardial infarction (MI) by 30% ($p<0.01$) in several large statin therapy trials in humans (4S, 1994, Shepherd *et al.*, 1995, Sacks *et al.*, 1996). This highlights the importance of cholesterol synthesis and its regulation in the development of CHD.

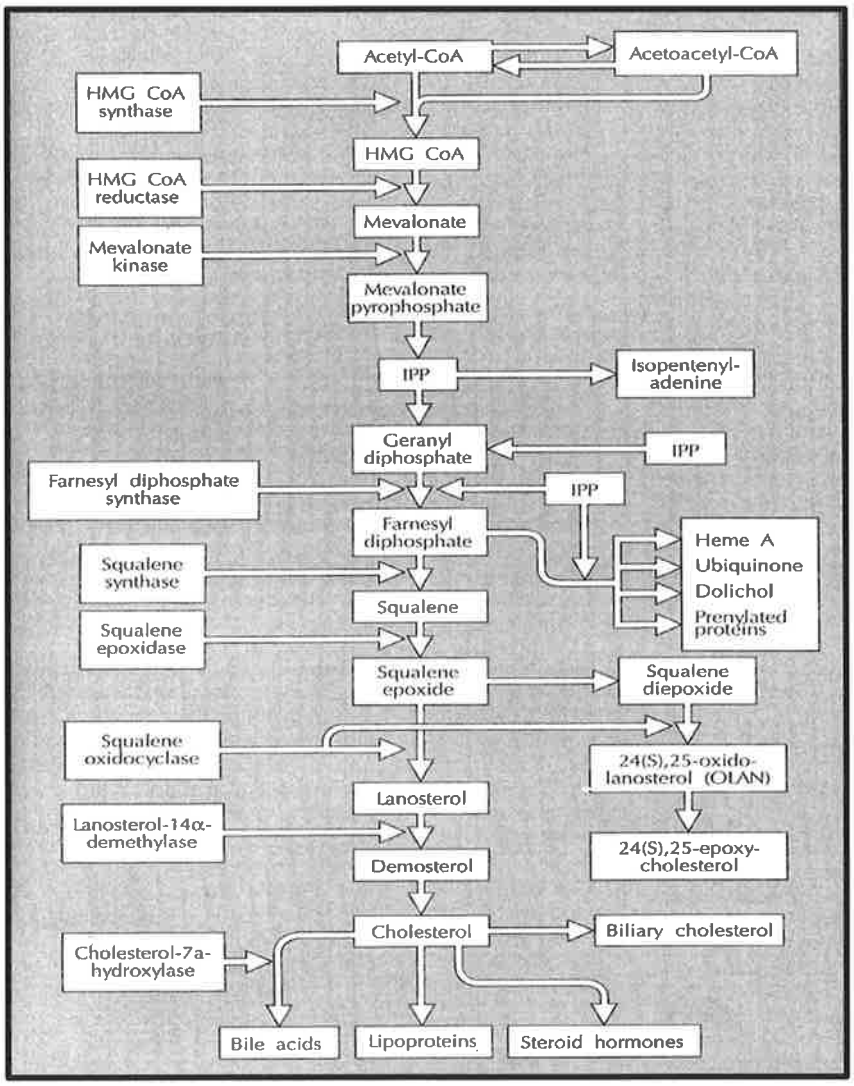


Figure 1.1 Pathways of Cholesterol Biosynthesis. Adapted from Rudney and Sankhavaram, (1993).

1.2.2 Cholesterol Esterification

Excess intracellular cholesterol can be stored within the cell as cholesterol esters. The esterification of cholesterol is catalysed by an enzyme called acyl : cholesterol acyltransferase (ACAT) which therefore plays an important role in cholesterol metabolism (Suckling and Stange 1985). Unesterified cholesterol within a cell can be potentially cytotoxic and therefore the conversion into the metabolically inert cholesterol esters protects the cells and prevents the degradation of membranes. When intracellular cholesterol levels are low the cholesterol esters can be hydrolysed back into unesterified cholesterol for use.

1.2.3 Cholesterol Catabolism – Bile Acid Synthesis

The formation of bile acids from cholesterol provides an important pathway by which excess cholesterol can be disposed of (Straka *et al.*, 1990). Two different pathways have been described for the synthesis of bile acids from cholesterol (Martin *et al.*, 1993, Schwarz *et al.*, 1997). The first pathway has been well characterised and involves a neutral or microsomal pathway, involving the 7α-hydroxylation of cholesterol by a microsomal cytochrome p450 enzyme called cholesterol 7α-hydroxylase, which is rate limiting. The second pathway is the mitochondrial pathway that involves the initial hydroxylation of cholesterol to 27-hydroxycholesterol (oxysterol) (Figure 1.2). This intermediate is then the substrate for a mitochondrial oxysterol 7α-hydroxylase. This enzyme has been proven to be distinct from cholesterol 7α-hydroxylase (Martin *et al.*, 1993) and has been isolated in pig liver mitochondria (Toll *et al.*, 1992, Axelson *et al.*, 1992).

The initial 27-hydroxylation of cholesterol to 27-hydroxycholesterol by 27-hydroxylase has been found to be an important pathway for the production of bile acids such as chenodeoxycholic acid and cholic acid. Cerebrotendinitus Xanthomatosis is a metabolic defect in which there is a lack of mitochondrial 27-hydroxylase activity. Patients with this disease have lower cholic acid synthesis, proving that 27-hydroxylation is a major pathway for cholic acid biosynthesis in man (Oftebro *et al.*, 1980). The enzyme also plays a major role in arterial cholesterol metabolism because these patients develop atherosclerosis at a very early age and often die from heart attacks before the age of 10 (Oftebro *et al.*, 1980).

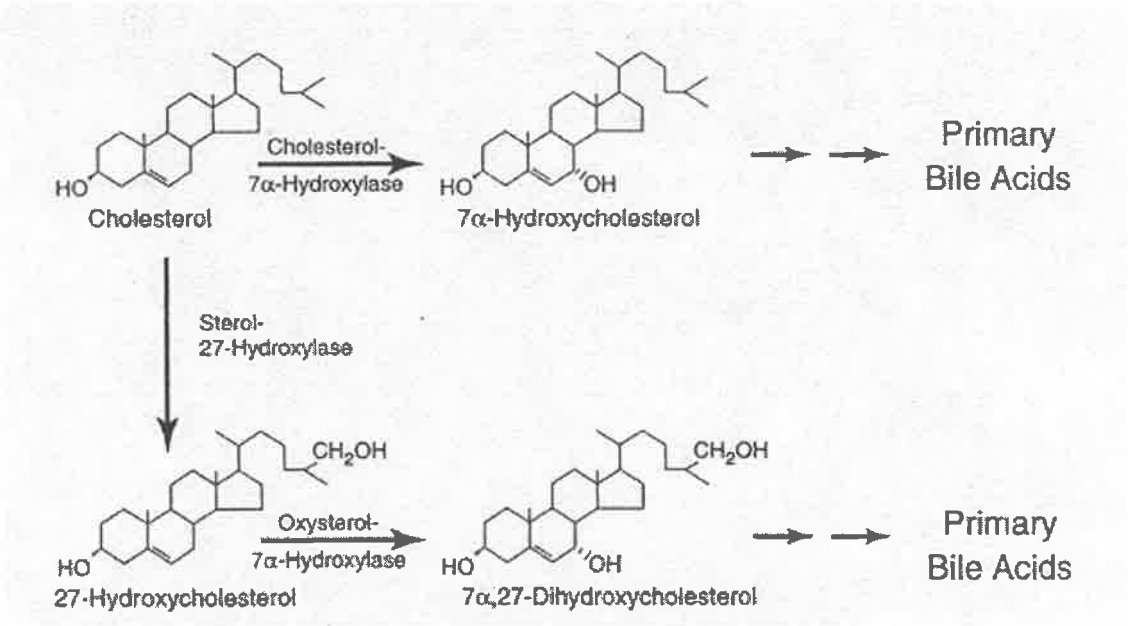


Figure 1.2. The two different pathways of bile acid synthesis: The "neutral" or microsomal pathway and the mitochondrial pathway, which involves the initial 27-hydroxylation of cholesterol. Adapted from Schwarz *et al.*, (1997) p 24000.

1.3 Lipids and Lipoproteins

The major lipids are unesterified and esterified cholesterol, triglycerides, phospholipids and free fatty acids (FFA). With the exception of FFA, these lipids are insoluble in water and they cannot be transported in the blood in their free form. Instead they are incorporated into amphiphatic molecules called lipoproteins. Lipoproteins are spherical structures composed of a hydrophobic core of esterified cholesterol and triglycerides surrounded by a surface monolayer of phosopholipid and unesterified cholesterol (Goldstein and Brown 1977). The protein moieties of lipoproteins are called apolipoproteins (apo) and are important because they regulate the interactions between lipoproteins and receptors or enzymes.

Lipoproteins are classified according to their density as determined by Havel *et al.* (1955) using ultracentrifugation. The major lipoprotein classes in increasing order of density are: chylomicrons ($d<0.95$ g/ml), very low-density lipoprotein (VLDL, $d<1.006$ g/ml), intermediate-density lipoprotein (IDL, $1.006<d<1.019$), low-density lipoprotein (LDL, $1.019 < d < 1.063$ g/ml) and high-density lipoprotein (HDL, $1.063<d<1.21$ g/ml). Each lipoprotein class has a different composition of lipids and apolipoproteins.

1.4 Lipoprotein Metabolism

1.4.1 Chylomicrons

Chylomicrons are triglyceride-rich lipoproteins that have a short half-life and are normally undetectable after an overnight fast. They are synthesised in the intestine and are responsible for transporting dietary triglyceride into the circulation (Thompson, 1990). In the small intestine, dietary fat is emulsified and hydrolysed by

the combined actions of pancreatic lipase and biliary secretions. The degradation products from this are incorporated to form water-soluble micelles and serve as precursors in triglyceride synthesis. Micelles allow the transport of lipids to the microvillus membrane where they diffuse across the epithelia. The majority of the re-synthesised triglyceride is then combined with cholesterol, phospholipids and protein to form chylomicrons (Symons, 1982). Once the chylomicrons have entered the circulation they pass into the peripheral circulation where they come into contact with an enzyme called lipoprotein lipase, located on the surface of the capillary endothelial cells. This enzyme hydrolyses triglycerides and smaller chylomicron particles are produced which are called chylomicron remnants (Symons, 1982, Goldstein and Brown 1977).

The majority of chylomicron remnants are removed from the circulation by the liver. This uptake can occur via a number of processes. There are interactions between the apoE moiety and the LDL receptor, which binds chylomicron remnants with a high affinity (Hui *et al.*, 1986). Other mechanisms, such as binding to the LDL receptor related protein (LRP) or heparin-sulphate-bound hepatic lipase, which also recognise apoE as the ligand, happen more slowly (Havel, 1995)

1.4.2 VLDL

VLDL shares many similar characteristics to chylomicrons except they are generally smaller, have slightly more cholesterol, phospholipid and protein. They have one major difference in that the triglyceride that they carry is predominantly endogenously produced.

The liver is the principal source of VLDL, but in fasting states the intestine secretes a VLDL sized particle containing triglycerides synthesised in the intestinal mucosa (Thompson 1990 and Symons 1982). Once hepatic VLDL is secreted from the liver it also comes into contact with lipoprotein lipase. This lipase hydrolyses triglycerides to fatty acids and glycerol (Bensadoun *et al.*, 1996). These fatty acids are delivered to adipose tissue for storage or muscles for energy production. Some of the VLDL remnant particles can then be cleared directly by the liver via the LDL receptor, which recognises both the ApoE and ApoB apolipoproteins present on VLDL remnants (Grundy 1991).

1.4.3 IDL

The further hydrolysis of triglycerides within the inner core of VLDL and the subsequent accumulation of cholesterol esters leads to the formation of intermediate-density lipoprotein (IDL). The IDL particle can then undergo two metabolic fates. Either it can be cleared directly by the liver, via the LDL receptor, or alternatively it may undergo further removal of triglycerides and apolipoproteins and accumulation of cholesterol esters leading to the formation of LDL (Brown and Goldstein 1986 and Havel 1984).

1.4.4. LDL

The main role of LDL is to transport cholesterol to the peripheral tissues. In humans it is the main cholesterol carrying lipoprotein, containing approximately 65% of the plasma's total cholesterol. This is not the case for other animals such as rats and rabbits who tend to carry the majority of their cholesterol in high-density lipoproteins (Daley *et al.*, 1994 and Roach *et al.* 1993). LDL is derived only from the VLDL, IDL

delipidation cascade described above. Its concentration depends on the balance of many different mechanisms, including the hepatic secretion of VLDL, its conversion from VLDL by lipoprotein lipase and the activity of LDL receptors. LDL has much less triglyceride than VLDL and IDL and its protein content is almost entirely ApoB ($ApoB_{100}$ in humans). The ApoB moiety is important as it allows the LDL particle to be recognised and bound by the LDL receptor. Once bound, LDL can then be cleared from the circulation via receptor-mediated endocytosis (Brown and Goldstein 1986).

As LDL is the main cholesterol carrying lipoprotein in human plasma it is also the most atherogenic (Rywik *et al.*, 1999). It has been found that when LDL cholesterol concentrations are increased by 10%, the risk of CHD subsequently increases by 20% (Rywik *et al.*, 1999). Diseases in which there are prolonged and elevated levels of LDL in the blood, such as nephrotic syndrome and diabetes mellitus, are often accompanied by premature or more severe atherosclerosis (Grundy 1997). Conversely, when LDL cholesterol concentrations are lowered between 24-50% using statin therapy, CHD deaths and MI are significantly reduced (-30%, 4S, 1994, Shepherd *et al.*, 1995, Sacks *et al.*, 1996).

1.4.5 HDL

In contrast to LDL, HDL is thought to be an anti-atherogenic lipoprotein. The HDL particle is synthesised either in the liver or in the intestine then enters the circulation initially as an immature, discoidal particle. However, it rapidly acquires lipids (unesterified cholesterol and phospholipid) from either cell membranes or LDL to become a mature, spherical structure. The cholesterol is esterified for this reaction by the plasma enzyme lecithin:cholesterol acyl transferase (LCAT). The major

phospholipid in HDL is phosphatidylcholine (also know as lecithin). It has an important functional role in the esterification of cholesterol, as the reactant in the enzymatic reaction catalysed by LCAT (Barter 1993).

HDL can be separated into two subclasses, HDL_2 and HDL_3, based on their different densities. The main apolipoproteins of HDL are apoAI and apoAII or both apoA variants (Cheung and Albers 1982). ApoAI has been shown to be an activator of the LCAT reaction.

Epidemiological studies have found that HDL cholesterol is inversely related to CHD (Gordon and Rifkind, 1989 and Kannel *et al.*, 1971). One mechanism to explain this is that HDL is believed to participate in a process termed "reverse cholesterol transport" (Barter 1993). This is the process by which cholesterol in the peripheral tissues is delivered to the liver by HDL, either for excretion from the body or for recycling. This process is believed to be anti-atherogenic because it has the potential to promote cholesterol efflux from the artery wall. In addition to this, reverse cholesterol transport is the only means for the elimination of cholesterol from cells in most tissues and is therefore important in maintaining cell-membrane homeostasis and normal cell function.

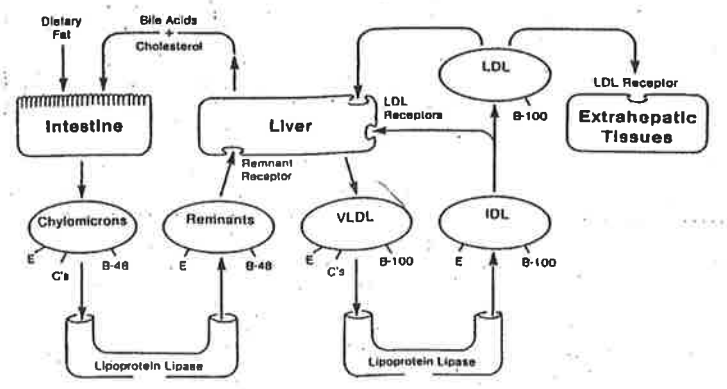


Figure 1.3. Lipoprotein Metabolism. Adapted from Beisiegel *et al.*, (1991) p190

1.5 Atherosclerosis

As previously mentioned, elevated plasma levels of LDL cholesterol are directly related to increased incidence of heart disease. Heart disease can manifest itself in many forms, the most common of which is atherosclerosis. Atherogenesis starts with the formation of a fatty streak that is initiated by an increased passage of LDL across the endothelium of an artery into its wall. This is likely to occur at sites of turbulence when LDL levels are high and when the endothelium is damaged by various mechanisms, for example, hypertension, oxidation or glycation (Ross and Glomset 1976, Ross 1981, Hunt 2000). Monocytes from the blood circulation are attracted to these sites by the damaged endothelium and cross the endothelium to enter the subintimal space. These monocytes can then take up LDL and assume the morphology of macrophages. Healthy, unmodified LDL is taken up slowly, if at all by macrophages. The LDL must undergo some modification, such as oxidation, before there is rapid uptake and foam cell formation is excited (Steinberg 1988).

Foam cells themselves can release growth factors that recruit smooth muscle cells located further out in the aortic wall into the fatty streak region. These smooth muscle cells differentiate into fibroblasts and lay down collagen over the foam cells, which then undergo either necrosis or apoptosis. This results in the formation of a pool of extracellular cholesterol ester trapped beneath a fibrous cap. This fibrous cap may eventually rupture if it becomes unstable, discharging the cholesterol from beneath it. Healing of this rupture may occur uneventfully but in some cases thrombosis may occur at the site of the ruptured cap and cause occlusion of the artery, resulting in myocardial infarction (Durrington and Sniderman 2000). In fatal cases, the lumen of

at least one major branch (usually 2 or 3) of a coronary artery is narrowed to less than 25% of its original diameter (Thompson 1990).

1.6 Oxidatively Modified LDL

1.6.1 Oxidation

In biological systems, oxygen is an important acceptor of electrons. This leads to the formation of active oxygen and free radical species. A free radical is any chemical species that has one or more unpaired electrons. Free radicals can perform important biological functions, for example, the nitric oxide radical is the endothelial-derived relaxation factor (EDRF) which relaxes smooth muscle cells. Many free radicals, however, are unstable and highly reactive and can become involved in unwanted reactions with biomolecules such as DNA, lipids and proteins, causing oxidative damage. This damage has been hypothesised to be a major contributor to aging and to many of the degenerative diseases of aging, including cardiovascular disease, cancer and the decline of the immune system (Singal *et al.*, 1998).

1.6.2 LDL Oxidation

LDL particles may also be modified by oxidation, which is thought to render it more atherogenic (Parthasarathy *et al.*, 1992). Oxygen free radicals are particularly reactive at the site of double carbon bonds in organic compounds and LDL has an abundance of these in the fatty acids of the phospholipids present in its outer envelope. Oxygen free-radical attack on these phospholipids leads to the formation of lipid peroxide products. These react with and damage apoB, the ligand for the LDL receptor (part of normal LDL metabolism) (Steinberg 1987). As a result these modified LDL particles

are no longer recognised by the LDL receptor. Instead they can be taken up rapidly by scavenger receptors, present on monocyte-derived macrophages, that may be present in the arterial wall in response to endothelial cell injury (Steinbrecher 1999). Unlike the LDL receptor, the scavenger receptor can not be downregulated when cholesterol enters the cells. Therefore, the uptake of modified LDL by the scavenger receptor causes the over-accumulation of cholesterol ester within these macrophages located in the arterial intima. This process then leads to foam cell formation, the hallmark of an atherosclerotic plaque (Ylitalo *et al.*, 1999) (See section 1.5).

LDL has its own defense mechanisms against oxidative damage, namely in the form of fat-soluble antioxidants. Ubiquinone, α-and β-tocopherol and β-carotene can dissolve in the central lipid core of LDL and react with the free radicals to neutralise their effects and offer protection. HDL may also protect LDL against oxidative modification as it has been found that HDL can metabolise the lipid peroxides before they undergo spontaneous breakdown to form apoB-damaging substances. The best way LDL oxidation can be decreased, however, is to reduce the concentration of LDL in the plasma.

The oxidation of LDL is thought to occur in the arterial intima rather than in the circulation because the plasma contains an abundance of water and lipid soluble antioxidants (thought to protect against oxidation). Measurement of LDL oxidation in the vessel wall, however, has not been well characterised and therefore the relevance of LDL oxidation *in vivo* has been questioned (Stocker 1994). Current *in vitro* methods of determining LDL oxidation generally involve isolating LDL, thereby taking it out of its native environment and perhaps do not therefore provide a valuable

reflection of what occurs in the vessel wall. A standard *in vitro* LDL 'oxidisability' test has been called for if it is to be used as an indicator of atherosclerotic risk.

1.6.3 In Vivo Oxidation of LDL and its Role in Atherosclerosis

Initial evidence that lipid peroxidation occurs *in vivo* emerged from studies which used immunostaining techniques to detect the presence of modified LDL in WHHL rabbit (Watanabe Heritable Hyperlipidemic rabbit-a rabbit deficient in LDL receptors) atherosclerotic lesions (Haberland *et al.*, 1988, Palinski *et al.*, 1989, Rosenfeld *et al.*, 1990). Antibodies can also be raised to specific epitopes present in oxidised LDL. These antibodies have been used to immunostain histological sections of aorta from WHHL rabbits and show the presence of oxidised LDL in atherosclerotic lesions but not in normal arteries (Palinski *et al.*, 1989). Also, in experiments conducted by Yla-Herttuala *et al.*, (1989), LDL extracted from human and rabbit atherosclerotic lesions, displayed many of the physiochemical and biological properties of *in vitro* oxidised LDL. These were, for example, increased electrophoretic mobility, increase in particle density, fragmentation of apoB, increase chemotaxis for monocytes and increased degradation of LDL by macrophages. The oxidised LDL from these lesions could also recognise the scavenger receptor of macrophages. Furthermore, the administration of antioxidants that prevent the oxidative modification of LDL have been shown to slow the progression of atherosclerosis (Steinberg 1988).

Whilst the experiments described above give proof that oxidised LDL plays a role in atherosclerosis, there still is a lack of direct evidence that distinguishes LDL oxidation as a consequence rather than a cause of atherosclerosis (Stocker 1999). This argument stems from the findings that α-tocopherol (the major antioxidant associated with LDL

and thought to be anti-atherogenic) is found in normal concentrations in human atherosclerotic lesions and can co-exist in these lesions with oxidised lipids (Suarna *et al.*, 1995, Niu *et al.*, 1999). In addition to this, it has been found that probucol (synthetic antioxidant) can substantially attenuate atherosclerosis in the aorta of apolipoprotein E -/- mice and cholesterol-fed ballooned rabbits without a concomitant inhibition of aortic lipid oxidation (Witting *et al.* 1999). Taken together, these findings cast some doubt as to whether lipid oxidation is a general cause of atherosclerosis.

In summary, there is evidence in the literature that *in vivo* LDL oxidation and lipid peroxidation is associated with the progression of atherosclerosis. Direct evidence, however, that LDL oxidation causes (rather than being a consequence of) atherosclerosis is still forthcoming.

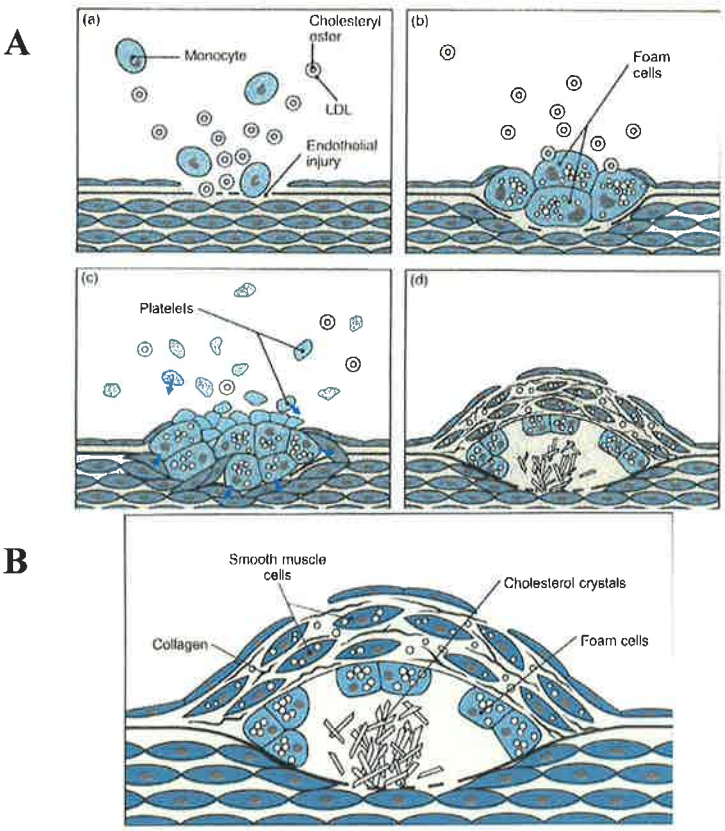


Figure 1.4. (A) Progression of atherogenesis following endothelial injury. (B) A diagrammatic representation of an atheromatous plaque. Adapted from Thompson (1990) p 90 and 92.

1.7 LDL Metabolism

1.7.1 The LDL Receptor Pathway

Goldstein and Brown first described the LDL receptor pathway in cultured human fibroblasts using ^{125}I-labelled LDL (Goldstein and Brown 1977). They described this pathway to consist of an ordered sequence of events in which LDL is first bound to a high-affinity receptor (i.e. the LDL receptor) on the cell surface and is then internalised by endocytosis and subsequently delivered to lysosomes for degradation.

The LDL receptor is a glycoprotein present on the outer surface of most cells and in particular liver cells. Its action in this pathway is such that it specifically recognises and binds to LDL particles via its single apoB protein. The LDL receptor can also recognise apoE containing lipoproteins, including chylomicrons, VLDL remnants, IDL and HDL. Some of these lipoproteins have multiple copies of apoE and also contain apoB. They therefore bind to the LDL receptor with a higher affinity than LDL itself, which is a result of being able to bind to multiple receptors (Brown and Goldstein 1986).

About 45 min after their synthesis, LDL receptors gather in clathrin coated pits on the outer surface of cells. When lipoproteins (LDL) bind to the LDL receptor, these coated pits invaginate to form coated endocytic vesicles in a process called receptor mediated endocytosis. Once internalised within the cell, the clathrin coat quickly dissociates leaving the remaining vesicle to fuse with others to form an endosome. Within the endosome the receptor dissociates from the LDL particle, a process believed to be promoted by acidification, and recycles back to the cell surface to bind

other lipoproteins. The LDL particle is then delivered to a lysosome where the proteins are hydrolysed to amino acids and the cholesterol esters are hydrolysed to cholesterol by an acidic lipase (Neindorf and Beisiegel 1991). The unesterified cholesterol liberated from LDL in this process mediates a complex series of feedback control mechanisms that protect the cell from an over-accumulation of cholesterol.

1.7.2 Regulation of the LDL Receptor

There are three main regulatory feedback events that occur when an LDL particle enters the cell and unesterified cholesterol is subsequently delivered to it (Goldstein and Brown 1977). Firstly, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase) is inhibited. This enzyme catalyses the rate limiting step in cholesterol synthesis and consequently less cholesterol is produced. Secondly, the LDL receptor is downregulated to decrease the influx of cholesterol into the cell and thirdly, there is an increase in ACAT, an enzyme that esterifies excess cholesterol for storage into cholesteryl droplets.

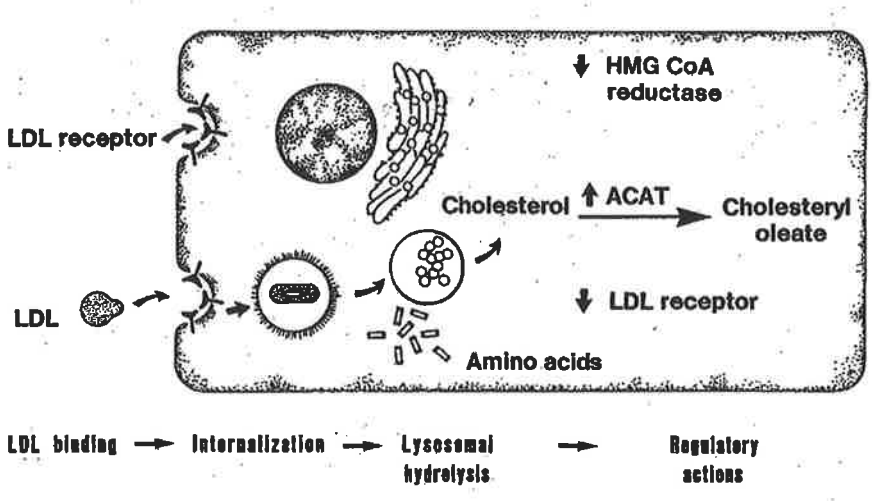


Figure 1.5. The LDL receptor pathway, showing the three main regulatory consequences of the delivery of unesterified cholesterol to the cell. Adapted from Beisiegel *et al.*, (1991) p191.

Whilst unesterified cholesterol appears to be the regulatory sterol in these feedback mechanisms, evidence from the literature suggests that oxygenated derivatives of cholesterol or what are termed "oxysterols" are actually the regulatory feedback effectors (Grundy 1991 and Haevekes *et al.*, 1987). These oxysterols have been found to possess far more potent downregulatory effects on both the LDL receptor (Takagi *et al.*, 1989) and HMGCoA reductase (Axelson *et al.*, 1995) than cholesterol itself.

The importance of oxysterols in the regulation of the LDL receptor was highlighted in a study by Takagi *et al.* (1989) that found 25-hydroxycholesterol downregulated the LDL receptor far more strongly than LDL cholesterol. Furthermore, when cells were incubated with ketoconazole, a substance that inhibits the formation of oxysterols, LDL no longer decreased the expression of the LDL receptor. However, the subsequent addition of 25-hydroxycholesterol to the ketoconazole-treated cells almost completely suppressed LDL receptor activity (Takagi *et al.*, 1989). This indicates that oxysterol formation is required for LDL receptor downregulation.

In another study by Axelson *et al.* (1995), it was found that the addition of LDL to normal fibroblasts, which were able to convert cholesterol to 27-hydroxycholesterol (the main endogenously formed oxysterol), decreased HMGCoA reductase activity by 73%. When 27-hydroxycholesterol formation was then selectively prevented by treatment with cyclosporin, the suppressive effects of LDL on HMGCoA reductase was reduced by a factor of 10. This also provides strong evidence that oxysterols are important regulatory feedback effectors in intracellular cholesterol metabolism.

1.7.3 Oxysterols

Oxysterols themselves, are sterols containing an extra hydroxy or ketone group at positions 7, 20, 25, and 27 (also referred to as 26) (Smith et al., 1996). They can enter the body through the diet or they can be produced endogenously both extra- and intra-cellularly. Outside cells, oxysterols are formed by free radical or oxidant attack on the cholesterol contained in lipoproteins. This forms various different types of oxysterols, the most common of which is 7-ketocholesterol (Patel *et al.*, 1996). These oxysterols can be taken up into cells and are directed predominantly to the liver (Lyons *et al.*, 1999). Intracellularly, oxysterols are formed by a mitochondrial p450 enzyme called 27-hydroxylase (Bellosta *et al.*, 1993). It converts the available unesterified cholesterol located in the "metabolically active pool" of unesterified cholesterol to 27-hydroxycholesterol, the main endogenously formed oxysterol. Although 25-hydroxycholesterol has been used commonly in studies and shown to be a potent downregulator of the LDL receptor and HMGCoA reductase, it may not be produced in sufficient quantities *in vivo* to be physiologically relevant. The formation of 27-hydroxycholesterol, however, appears to be more relevant because it is present in human plasma at higher concentrations (Javitt *et al.*, 1981). It has also been found to have potent downregulatory effects of the LDL receptor and cholesterol synthesis (Corsini *et al.*, 1995) making it a more likely physiological effector.

As mentioned above, the unesterified cholesterol available for conversion to 27-hydroxycholesterol is thought to be situated in a "metabolically active pool" of unesterified cholesterol within the cell. The location of this pool, however, is not known. The size of this pool can be affected by many factors including the hepatic production of lipoproteins, the conversion of cholesterol into bile acids, cholesterol

synthesis and the esterification of cholesterol. The net result of all these various inputs and outputs of cholesterol governs the size of this active pool of free cholesterol which in turn will regulate the activity of the LDL receptor, via the formation of these regulatory oxysterols (Grundy 1991).

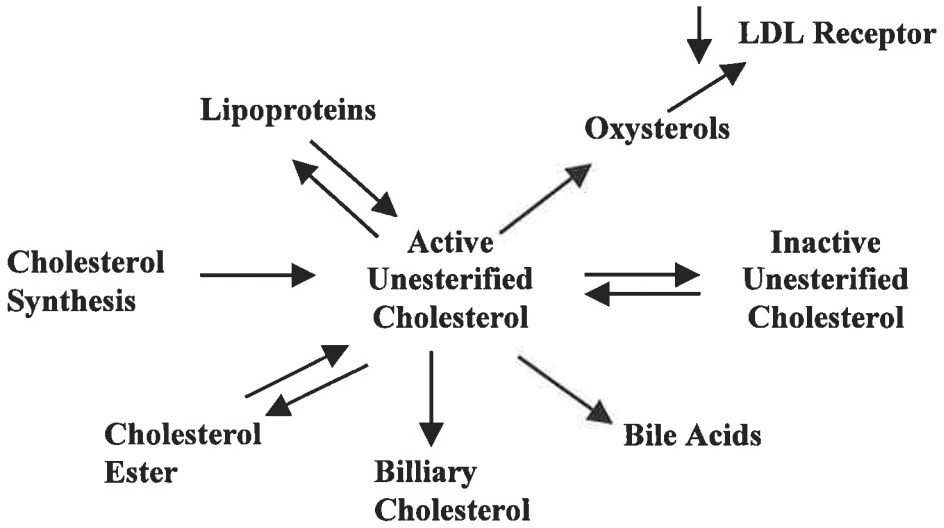


Figure 1.6. Diagram of factors regulating the intrahepatic concentration of active unesterified cholesterol. The latter gives rise to oxysterols, which in turn downregulate the synthesis of LDL receptors. Adapted from Grundy, (1991).

1.8 The LDL Receptor

1.8.1 Importance of the LDL Receptor

The main role of the LDL receptor is to remove cholesterol-carrying LDL from the circulation. The importance of this mechanism is highlighted in patients with genetic aberrations in the LDL receptor pathway who have accelerated atherosclerosis and heart attacks early on in life. Familial Hypercholesterolaemia (FH) is inherited as an autosomal dominant trait and exists clinically in two forms, either the heterozygote or

the more severe homozygote form. LDL consequently accumulates in the blood, increasing the person's risk for developing atherosclerosis and CHD (Goldstein and Brown 1975). The concentration of LDL cholesterol in these individuals is 2-3 fold higher in heterozygotes and 4-6 fold higher in homozygotes. Homozygotes often can have heart attacks before the age of 10 if untreated. FH can result from four different classes of mutation in the LDL receptor. These different types of mutations affect different steps in the LDL receptor pathway including: 1. failure to synthesis LDL receptors (most common), 2. failure to be transported from the endoplasmic reticulum to the golgi complex, 3. failure to bind LDL normally and finally 4. failure to cluster in coated pits (Brown and Goldstein 1986).

The Watanabe heritable hyperlipidemic rabbit (WHHL) is a strain of rabbits that have extremely elevated plasma cholesterol levels and are very prone to atherosclerosis. They develop severe atherosclerosis within the first few months of life followed by CHD. The WHHL has a class 2 genetic defect in the LDL receptor gene and consequently cholesterol is removed from the plasma at a reduced rate and lipid levels are elevated (Watanabe 1980).

Both the FH and WHHL genetic models emphasise the importance of the LDL receptor in regulating plasma LDL-cholesterol levels and preventing CHD. In addition to this, more recently LDL receptor knockout mice have been produced which also exhibit dramatically increased plasma LDL levels (Sjoland *et al.*, 2000).

1.8.2 Structure

The LDL receptor is a single pass membrane protein that is initially synthesised as a precursor of apparent molecular weight on an electrophoresis gel of MW 120,000 Dalton's. It is synthesised in the rough endoplasmic reticulum and converted to a protein of 164,000 Daltons in the golgi apparatus by the addition of carbohydrate before being inserted into cell membranes (Schneider *et al*, 1982, Gianturco et al., 1987). The receptor is a multidomain protein, containing five distinct domains. The first domain of the LDL receptor consists of 292 amino acids and is located on the external surface of the cell membrane. It contains seven repeats of 40 amino acids and within each of these repeats there are six cysteine residues. These cysteine residues are disulphide bonded making it a tightly cross-linked structure. This aids its stability and helps to maintain its binding activity. This domain also contains clusters of negatively charged amino acids at one end of the repeats. These are believed to be the binding sites of the LDL receptor that will bind to the positively charged regions of its ligands (apoE and apoB).

The second domain consists of approximately 300 amino acids and is 35% homologous to the extracellular domain of epidermal growth factor (EGF). This region is required for the disassociation of the receptor from its ligand and the recycling of the receptor to the cell surface. The third domain is rich in threonine and serine residues in a total of 58 amino acids. Its importance is still yet to be elucidated, as deletion of this region does not effect LDL receptor function in any way. The fourth domain is composed of 22 hydrophobic amino acids. It is the membrane-spanning domain of the LDL receptor and is required to anchor the receptor into the cell membrane. Lastly, the fifth domain of the LDL receptor is the cytoplasmic tail

and contains the carboxy terminus. This region is important for the clustering of the receptor into clathrin coated pits. This was determined from molecular analysis that found three separate mutations which prevented the proper formation of the cytoplasmic tail and consequently the receptors did not cluster into clathrin coated pits (Goldstein and Brown 1977).

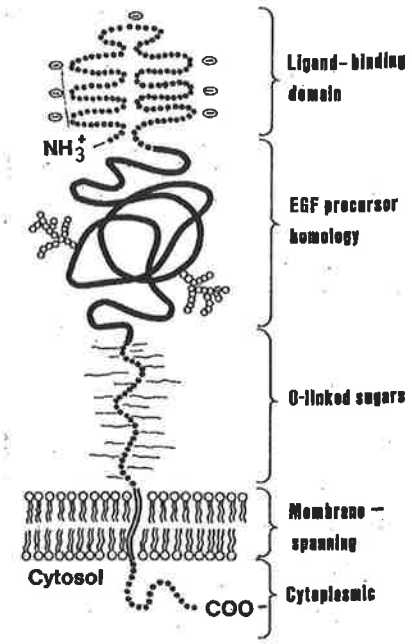


Figure 1.7. Structure of the LDL Receptor protein including the five important structural domains for the receptor. Adapted from Beisiegel et al., (1991) p190.

1.8.3 LDL Receptor Gene and its Regulation

The LDL receptor gene is located in bands p13.1-13.3 in the distal short arm of chromosome 19. It is approximately 50 kb long and consists of 18 exons which are separated by 17 introns. Expression of the LDL receptor is tightly regulated at the level of gene transcription in order to maintain an optimal concentration of cholesterol within the cell. The LDL receptor is able to be upregulated and downregulated depending on the cell's cholesterol requirements.

Oxysterols, namely 27-hydroxycholesterol, are thought to be the regulatory sterols that downregulate the LDL receptor (Section 1.7.2). Oxysterols have been found to downregulate the LDL receptor by inhibiting the cleavage of two specific transcription factors called sterol regulatory element binding proteins (SREBPs) from the endoplasmic reticulum (Winegar *et al.*, 1996). When cholesterol concentrations within the cells are low and hence oxysterol concentrations low, these SREBPs can be cleaved from the membrane of the endoplasmic reticulum. This cleavage releases the mature active transcription factor form of SREBP that can travel to the nucleus, bind upstream of the LDL receptor gene and can activate transcription (Briggs *et al.*, 1993) (Section 1.9.2).

1.9 Sterol Regulatory Element Binding Proteins (SREBPs).

1.9.1 Stucture

SREBPs are encoded by two genes designated SREBP-1 and SREBP-2. The SREBP-1 gene gives rise to two transcripts called SREBP-1a and 1c whose functions do not appear to be distinctly different. These binding proteins are orientated in a hairpin fashion on membranes of the endoplasmic reticulum (Hua *et al.*, 1995). Both their amino terminal segment (500 amino acids) and carboxy terminal segment (590 amino acids) project into the cytoplasm. A membrane attachment domain, which projects into the lumen of the endoplasmic reticulum, joins these segments. The amino-terminal segment contains the basic helix-loop-helix-leucine zipper motif and the transcription-activating domain (Brown and Goldstein 1997).

1.9.2 SREBP Activation

As mentioned in Section 1.8.3, SREBPs are cleaved to their mature active form when cholesterol concentrations are low to increase the LDL receptor. This cleavage of SREBPs to their mature form occurs via proteolytic processing in a two step fashion (Sakai *et al.*, 1996). Firstly, a sterol sensory protein called SREBP cleavage-activating protein (SCAP) is activated via an unknown sterol/SCAP interaction (Nohturfft *et al.*, 1999). This interaction then allows SCAP to transport SREBP's to the post ER compartment and form a tight complex near the membrane bound "Site 1 protease" (S1P). Formation of this complex is required for the first proteolytic cleavage at "site 1" which is located in the lumenal loop (Cheng *et al.*, 1999). This cleavage separates the amino-terminal and carboxy-terminal segments but both segments remain attached to the lumenal membrane via their trans-membrane domains. The next step in this process then happens automatically via a second protease, termed site 2 protease (S2P), which cleaves the amino-terminal segment somewhere in its trans-membrane domain called site 2 (Zelenski *et al.*, 1999). This second proteolytic cleavage step appears to be non-regulated and immediately follows on from the first cleavage (Brown and Goldstein 1999). This releases the amino-terminal segment to produce the mature active transcription factor form of SREBP which can travel to the nucleus and activate transcription of genes containing sterol regulatory elements (SREs) in their promoter region (Briggs *et al.*, 1993). SREs are cis-acting promoter elements located upstream of the promoter region of genes encoding the LDL receptor, multiple enzymes of cholesterol biosynthesis including HMGCoA reductase and triglyceride biosynthesis including acetylCoA carboxylase (Brown and Goldstein 1999).

1.9.3 Independent Regulation of SREBP-1 and -2

In a variety of cultured liver cells, including HepG2 cells, SREBP expression has been found to exist (Brown and Goldstein, 1997). In these cells the proteolytic processing of both SREBP-1 and SREBP-2 appears to be regulated in parallel i.e. they are both cleaved when the cells are deprived of sterols and their cleavage is suppressed in the presence of sterols.

In hamsters and mice, however, it appears that SREBP-1 and SREBP-2 are regulated independently. Animal studies have found that when hamsters are fed their normal low fat chow diet SREBP-1 is present in their liver nuceli but there is little SREBP-2 (Sheng *et al.*, 1995). This suggests that SREBP-1 regulates the basal transcription of the LDL receptor and cholesterol synthesis. However, when hamsters are fed mevinolin (inhibitor of cholesterol synthesis) along with this low fat diet, it leads to an increase in the mature active form of SREBP-2 and a concurrent decrease in SREBP-1 (Sheng *et al.*, 1995, Shimomura *et al.*, 1997). This was also accompanied by a marked increase in the mRNAs for cholesterol related genes, including HMGCoA reductase and the LDL receptor. These findings indicate that whilst SREBP-1 is responsible for the basal transcription of the LDL receptor and cholesterol synthesis, SREBP-2 appears to be involved in the increases that occur when sterols are depleted.

Studies have also found that SREBP-1 is involved in the regulation of fatty acid metabolism but SREBP-2 is not. In support of this, Xu *et al.* (1999) found that fatty acids suppressed the mature form of SREBP-1 and did not affect SREBP-2. SREBP-1a has also been found to stimulate the transcription of the gene encoding acetylCoA carboxylase, which provides the malonyl CoA substrate for fatty acid synthesis.

SREBP-1c was cloned independently from rat adipocytes and was designated adipocyte differentiation factor (ADD-1) (Tontonoz *et al.*, 1993). Furthermore, in transgenic mice overexpressing SREBP-2, cholesterol synthesis is activated in preference to fatty acid synthesis (Horton *et al.*, 1998). Overall however, it has been suggested that whilst SREBP-1 may play an auxiliary role in fatty acid biosynthesis under certain conditions, other factors can replace it under normal conditions (Brown and Goldstein 1997).

In summary, the available literature suggests that *in vitro* SREBP-1 and –2 are regulated in parallel, whilst *in vivo* they appear to be regulated independently. SREBP-1 may also have a role in fatty acid metabolism but its importance in this process is unclear.

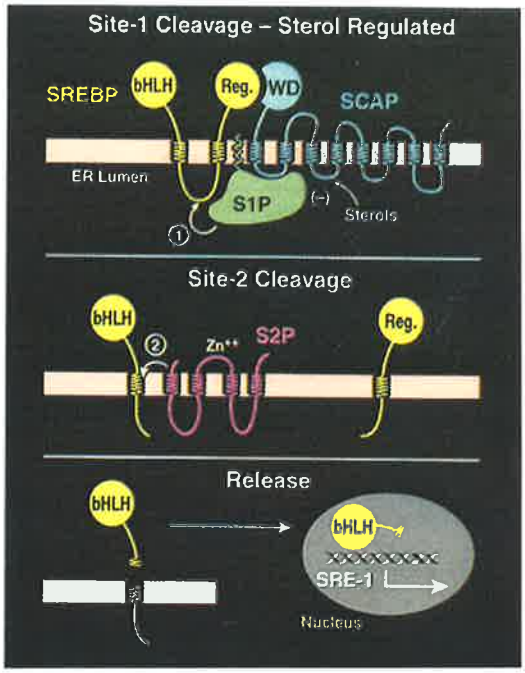


Figure 1.8. Model for the sterol-mediated proteolytic release of SREBPs from the membrane of the endoplasmic reticulum. Release is mediated by Site-1 protease (S1P), a sterol-regulated protease that recognises the SCAP/SREBP complex and cleaves SREBP in the luminal loop. After this cleavage, the Site-2 protease (S2P) cleaves the NH2-terminal bHLH-Zip domain of SREBP at a site located within the membrane-spanning region. This second cleavage releases the mature active form of SREBP, which can then travel to the nucleus where it activates genes controlling lipid synthesis and uptake. Adapted from Brown and Goldstein (1999) p 11042.

1.10 Antioxidants

Antioxidants, by definition are "any substance that when present at low concentration compared to the oxidisable substrate, significantly delays or prevents oxidation of the substrate" (Halliwell 1990). Antioxidants are able to "neutralise" free radicals by three main mechanisms: 1) they can either act as scavengers of free radicals (eg mannitol, superoxide dismutase, catalase and glutathione), 2) chain-breaking antioxidants (eg α-tocopherol, ascorbic acid, probucol) which react with intermediate peroxyl radicals and 3) preventative antioxidants (haem, transferrin, albumin and caeruloplasmin) which act by binding metal ions thereby preventing metal ion-catalysed production of free radicals.

Antioxidants are also distinct in terms of their lipid solubility. Some antioxidants are lipophilic enough to be transported in LDL particles (α-tocopherol, β-carotene and lycopene). Others are hydrophilic and are transported around in the plasma (e.g. vitamin C and catechins) where they are capable of preventing the oxidation of circulating LDL.

1.11 Green Tea and its Antioxidants

1.11.1 The Catechins

Green tea is a traditional Asian beverage that is an extract of the plant *Camellia sinensis*. It is derived from the same tea plant as black tea but it has not gone through the same fermentation process. In the production of green tea, leaves are heated at high temperatures immediately after plucking. This inactivates an oxidative enzyme, called polyphenol oxidase, contained in the plant leaf and released by plant cell

rupture. For black tea preparation, the leaves are extensively macerated which releases the oxidative enzymes to ensure maximal contact between the enzymes, the polyphenols and the atmospheric oxygen. This oxidation process is then allowed to proceed for 45-90 min (Graham 1992).

These different preparative procedures mean that green tea and black tea are composed of vastly different constituents. Green tea contains an abundance of potent antioxidants called catechins (Harbowy and Balentine, 1998). There are four main catechins in green tea including (-) epicatechin [EC], (-) epigallocatechin [EGC], (-) epicatechin gallate [ECG] and (-) epigallocatechin gallate [EGCG]. Of these, EGCG is the most abundant catechin in green tea as well as the most potent antioxidant (Jovanovic *et al.*, 1995, Nanjo et al., 1999, Kondo *et al.*, 1999). In addition to their antioxidants properties, catechins have been found to exhibit hypocholesterolaemic (Chan *et al.*, 1999, Yang and Koo 2000), antimutagenic (Imai *et al.*, 1997, Fujiki *et al.*, 1999) and antibacterial effects (Ikigai et al., 1993).

Structurally the catechins are characterised by multiple hydroxyl groups on two benzene rings. The gallo catechins have one extra hydroxy group in the 5' position on what is called the B ring, while the catechin gallates have an extra benzene ring with three more hydroxyls (see Figure 1.9).

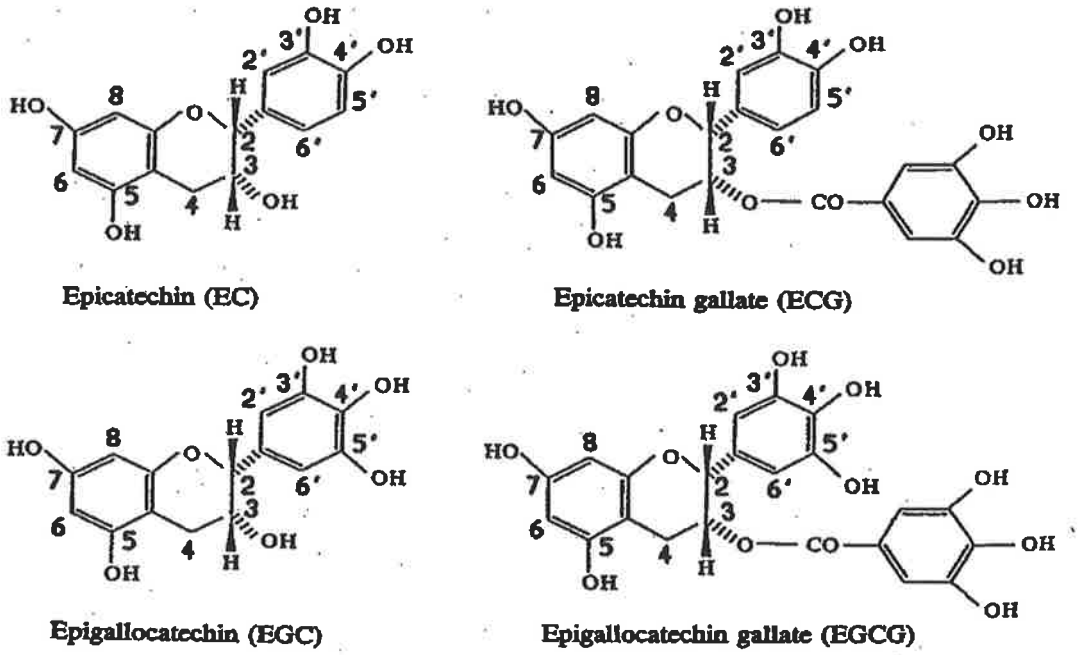


Figure 1.9. Chemical structures of the four main catechins in green tea.

In a normal infusion of green tea these catechins constitute approximately 30% of total dry weight solids (Harbowy and Balentine 1997). In contrast to this, black tea contains a far smaller amount of catechins (9% dry weight) due to the oxidation process that black leaves experience in their preparation. This process produces many different complex molecules, the majority of which are theaflavins and thearubigens. As with green tea, these compounds in black tea have been found to exhibit antioxidant properties (Graham 1992, Yoshino *et al.*, 1999) as well as hypocholesterolaemic (Matsumoto *et al.*, 1998) and antimutagenic (Shiraki *et al.*, 1994) effects.

Catechins are found in their highest quantities in green tea but they can also be found in various fruits and vegetables (Arts *et al.*, 2000a), red wine (Arts *et al.*, 2000b), and

chocolate (Arts *et al.*, 1999). The daily consumption of these catechins is extremely variable depending on food habits and may range from 25 mg/d to 1 g/d (Manach *et al.*, 1999).

1.11.2 Metabolism of Catechins

Due to the many biological effects of these catechins it is important to understand their bioavailability and metabolism. This has been investigated in several animal and human studies. In rats, oral administration of purified EGCG resulted in peak plasma levels of EGCG after 1 h, which then reduced to undetectable levels after 2 h (Unno and Takeo 1995). In human studies, plasma concentrations of EGCG, EGC and EC peaked between 1.4 and 2.4 h after oral administration (Yang *et al.*, 1998, Pietta *et al.*, 1998). The amount of catechin absorbed corresponded to 0.2-2% of the total catechin consumed. When the concentration of catechin was increased their peak plasma concentrations appeared to increase in a dose response fashion (Nakagawa *et al.*, 1997). In contrast to this, Yang *et al.*, (1998) witnessed a saturation effect, not a clear dose-response relationship.

EGCG has been found to be the most effectively absorbed catechin, followed by EGC and EC (Yang *et al.*, 1998). EGCG was also found to have the longest half-life of the catechins (5-5.5 h compared with 2.5 –3.4 h).

Catechins have been found to undergo various metabolic changes in the body. Studies have found that the catechins are extensively glucuronidated and this occurs initially in the intestinal mucosa where the activity of uridine-5'-diphosphoglucurinosyltransferase (UGT) is at its highest (Terao, 1999). Catechins can

then enter the blood circulation exclusively in the glucuronized form where they are directed predominantly to the liver to become either sulphated or methylated (Piskula and Terao, 1998). Glucuronidation tags the catechin compounds to be excreted via the bile and sulphation tags them for the urine. It has not yet been determined conclusively if methylation of the catechins tags them for the bile or the urine. The catechins are also directed to other tissues such as the brain, kidney, lung and heart, as their presence has been detected in these organs one hour after ingestion (Nakagawa *et al.*, 1997, Suganuma *et al.*, 1998). The majority of orally administered catechin, however, remains in the intestine.

In summary, orally ingested catechins can be absorbed through the intestine and are present in the common blood circulation and tissues in the form of various metabolites. Despite not having a long half life (4-5 h), they evidently remain in the circulation long enough to produce biological effects in humans and animal models (See section 1.12).

1.11.3 Antioxidant Properties of the Catechins

The most common property of these polyphenolic compounds is their antioxidant activity which is at least 2 fold greater than other well know dietary antioxidants such as α-tocopherol and β-carotene (Jovanovic *et al.*, 1995, Rice-Evans *et al.*, 1996). Their antioxidant potential is closely linked to their number of hydroxyl groups – in general, the higher the number the more potent the antioxidant action of the compound. Therefore EGCG (8 hydroxyls) has been found to exhibit the greatest antioxidant activity, closely followed by ECG (7 hydroxyls), EGC (6 hydroxyls) and EC (5 hydroxyls) using pulse radiolysis (Jovanovic *et al.*, 1995, and Nanjo *et al.*,

1999) (Figure 1.9). The antioxidant action of catechins has also been reproduced *in vivo* in rats. For example, when rats drank a 2% (w/v) green tea infusion it protected against liver peroxidation when treated with the oxidant 2-nitropropane (Hasegawa *et al.*, 1995). The administration of similar green tea extracts has also been found to increase the antioxidant capacity of rat plasma (Da Silva *et al.*, 1998, Terao, 1999). In humans, it has been found that drinking a single cup of green tea (300 ml) after an overnight fast elevated the antioxidant capacity of the blood (Serafini *et al.*, 1995). Similar findings were also found when volunteers consumed a single dose of green tea solids (2g) mixed with hot water (Leenen *et al.*, 2000, Benzie *et al.*, 1999).

As the majority of ingested catechins are modified in some way (see Section 1.11.2), and animal and human intervention studies have found that green tea and its catechins can increase the antioxidant activity of the plasma, it suggests that the main metabolites of the catechins must also possess antioxidant activity. This has been investigated in a couple of studies. Harada *et al.* (1999) found that glucuronide conjugates of the catechins exhibited high antioxidant activities. They were also present in the rat plasma in high quantities, far higher than the original ingested catechin compounds. It was suggested that these metabolites would be largely responsible for the antioxidant defense character added to the plasma by consumption of catechin containing foods. Manache *et al.* (1999) also presented proof that catechin glucuronidated conjugates were effective antioxidants. These conjugated derivatives had the same electrochemical behavior across an electrode array as the original catechins compounds, which is representative of their oxidation potential and hence their antioxidant capacity.

1.11.4 Antioxidant Action of Catechins

As mentioned previously, catechins are polyphenolic compounds that act as antioxidants by scavenging free radicals via the donation of a phenolic hydrogen group, which inactivates the free radical species. The polyphenol's subsequent oxidation leads to the formation of phenoxide ions that contain oxygen atoms with unpaired electrons. Delocalisation of the electron to a carbon atom stabilises this compound which may then react with the other radicals forming C-C bonds or C-O bonds. In this way oxidation of phenols leads to the formation of polymeric products (Rice-Evans *et al.*, 1996). The most important feature of the catechin structure, with regard to its antioxidant activity, is the ortho-dihydroxy catechol (3',4'-OH) arrangement on the B ring (Figure 1.9). This arrangement is thought to promote the formation of a stable phenoxyl radical and participates in electron delocalisation. Support for the importance of this 3',4'-OH arrangement on the B ring of polyphenols was highlighted in a study where the 3'-OH group was eliminated and its absence consequently reduced the antioxidant activity of the catechin compound to 27% of its original activity (Rice-Evans et al., 1996).

In addition to acting as efficient free radical scavengers, polyphenols have a strong affinity for divalent ions such as Cu (II) and Zn (II) and may also exert antioxidant activity by chelating these transitional metal ions which could otherwise initiate metal –catalysed lipid oxidations (Salah *et al.*, 1995).

1.12 Green Tea, Catechins and Atherosclerosis

1.12.1 Effects on LDL Oxidation

As with other dietary antioxidants, such as vitamin C, α-tocopherol and β-carotene, green tea and its catechins are thought to be anti-atherogenic compounds due to their ability to inhibit LDL oxidation, a possible initiating mechanism in the formation of atherosclerotic plaques. Numerous *in vitro* studies have found that in the presence of all the different catechins or several green tea extracts, the lag time of LDL oxidation is significantly increased after Cu^{2+}-induced and cell-mediated oxidation (Miura *et al.*, 1994, Zhang *et al.*, 1997, Pearson *et al.*, 1998, Yang and Koo, 1999, Yoshida *et al.*, 1999). This effect of catechins has also been demonstrated *in vivo* in rats (Hayek *et al.*, 1997) and human plasma (Lotito and Fraga 1998) where the addition of catechins to plasma samples increased LDL oxidation lag time and delayed the formation of thiobarbituric acid-reactive substances (TBARS). Furthermore, in human *ex vivo* studies, the plasma from volunteers who drank 4 cups of green tea also had an increased lag time to oxidation (Wang *et al.*, 1999, Burke *et al.*, 2000).

1.12.2 Hypocholesterolaemic Action of Green Tea and Catechins

Just as a reduction in LDL oxidation may protect against the development of atherosclerosis, so too may a reduction in plasma cholesterol concentrations. Epidemiological studies in Asia have found that there is an inverse relationship between drinking green tea and total serum cholesterol concentrations (Kono *et al.*, 1992, Imaj *et al.*, 1995, Kono *et al.*, 1996). These studies found that people who consumed five to ten cups of green tea daily, had significantly lower serum cholesterol levels (up to 8 mg/dl) compared to those who did not consume any green tea. In addition to this, green tea drinking was found to be inversely related to LDL

cholesterol concentration (Imaj *et al.*, 1995, Kono *et al.*, 1996) and positively related to HDL cholesterol (Imaj *et al.*, 1995).

Intervention studies in rats, mice and hamsters have also found that green tea and green tea extracts enriched in catechins exhibit hypocholesterolaemic effects (Muramatsu *et al.*, 1986, Matsuda *et al.*, 1986, Yang and Koo 1997, Chan *et al.*, 1999 and Yang and Koo 2000). For all of these studies the animals were initially made hypercholesterolaemic by feeding them either cholesterol (1% w/w) and/or lard (15% w/w) mixed in with their normal chow for a period of 1-4 weeks. The animals were then fed various green tea extracts enriched in catechins, which were either mixed in with their food or added to their drinking water, or freshly brewed green tea for a period of 4-8 weeks. After treatment with these green tea extracts, plasma total cholesterol concentrations were significantly reduced by 20-40%, compared to controls. A significant improvement in the atherogenic index was also noted in these studies (Muramatsu et al., 1986, Yang and Koo 1997). One study found that treatment with green tea significantly lowered apoB concentrations, indicating that LDL cholesterol may have been lowered (Chan *et al.*, 1999) but no changes were found in HDL cholesterol for any of the studies.

The most dramatic changes in plasma lipids occurred in a study by Fukuyo *et al.*, (1986) where 1% (w/w) pure EGCG was administered to rats for a period of 4 weeks. This resulted in a 50% decrease in plasma cholesterol concentrations as well as a 68% decrease in LDL cholesterol. There was also a significant increase in HDL cholesterol (+40%) and a reduction in triglycerides (-20%). This last study indicates that perhaps EGCG alone has more potent hypocholesterolaemic effects than green tea or the other

catechins combined. In contrast to these findings, Tijburg *et al.*, (1997) found that a green tea extract, included in the drinking water for 21 weeks, did not significantly decrease cholesterol concentrations in the cholesterol-fed hypercholesterolaemic rabbit.

Human intervention studies investigating the effects of green tea on plasma lipids are limited. A trial by van het Hof *et al.* (1997) however, found that the consumption of 6 cups (900 ml) of green tea for 4 weeks did not significantly alter plasma lipids. Another study (Princen *et al.*, 1998) also found that when 29 volunteers consumed 900 ml of green tea per day for 4 weeks it did not alter plasma lipids nor did the administration of a green tea polyphenol supplement, equivalent to 18 cups of green tea per day. One possible reason for the lack of hypercholesterolaemic effect of these green tea treatments may be that 4 weeks is not a sufficient period of time to produce a change. In addition to this, the subjects in these studies were normolipidaemic and therefore a reduction in plasma cholesterol concentrations may have been harder to achieve.

1.12.3 Mechanisms by which Green Tea and its Catechins may Lower Plasma Cholesterol

Cholesterol Absorption

Inhibition of cholesterol absorption has been proposed as a mechanism to explain the cholesterol-lowering effects of green tea. This is because the faecal excretion of total lipids and cholesterol were found to be higher in animals consuming green tea extracts (Muramatsu *et al.*, 1986, Fukuyo *et al.*, 1986, Matsuda *et al.*, 1986, Chan et al., 1999). The EGCG has also been observed to inhibit the uptake of ^{14}C-cholesterol

from the intestine (Chisaka *et al.*, 1988). This apparent reduction in intestinal absorption has been ascribed to EGCG reducing the solubility of cholesterol into mixed bile salt micelles (Ikeda *et al.*, 1992). It has also been found recently that hamsters and rats fed green tea extracts had increased faecal excretion of bile acids.

Cholesterol Synthesis

A reduction in cholesterol synthesis is another mechanism by which green tea and its catechins could lower plasma cholesterol concentrations. Studies by Chan *et al.* (1999) and Yang and Koo (2000) have found no effect of green tea on the "*in vitro*" activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase in hamsters and rats. The HMGCoA reductase enzyme catalyses the rate-limiting step in cholesterol biosynthesis, but measurement of its activity "*in vitro*" may not always reflect the level of cholesterol synthesis. For example, treatment with inhibitors of cholesterol synthesis (e.g. the statins) have been found to elevate, not decrease, "*in vitro*" HMGCoA reductase activity (Kovanen *et al.*, 1981, Kita *et al.*, 1980). More direct measures of cholesterol synthesis such as the incorporation of tritium in cholesterol using tritiated water (Carrella *et al.*, 1999) or the plasma ratio of lathosterol to cholesterol (Roach *et al.*, 1993) have confirmed that whole body cholesterol synthesis is, in fact, lowered with statin treatment. This is despite often marked increases in "*in vitro*" HMGCoA reductase activity (Kovanen *et al.*, 1981, Kita *et al.*, 1980).

LDL Receptor

Another mechanism by which green tea and its catechins could lower plasma cholesterol concentrations is to increase the LDL receptor. Indirect evidence that the LDL receptor may be upregulated by green tea extracts was found in a study by

Chisaka et al. (1988). When rats were fed EGCG, the removal of intravenously injected ^{14}C-cholesterol from the plasma was enhanced. This increase in the plasma clearance of cholesterol may have been due to the upregulation of the LDL receptor as it is the main mechanism by which sterol is removed from the circulation (Dietschy and Wilson, 1970). In support of this, studies in our laboratory have found that freshly brewed green tea can increase the LDL receptor in cultured human hepatoma (HepG2) cells. Furthermore, the administration of a crude catechin extract from green tea increased the hepatic LDL receptor in rats (See Section 1.13).

1.12.4 Effects on Atherosclerotic Lesion Formation

The ability of green tea and its catechins to inhibit LDL oxidation (Section 1.12.1) and lower plasma cholesterol concentrations (Section 1.12.2) suggests that they may have the potential to reduce the development of atherosclerosis and hence CHD. Support for this has been found in animal studies where fatty streak formation was measured using lipophilic staining with oil red O. Xu *et al.* (1998) found that the administration of 200 mg of catechin/day for 16 weeks reduced fatty streak formation in rabbits by 30%. Similar findings were found in a study by Hayek *et al.* (1997) where 50 μg of catechin was fed daily to rabbits for 6 weeks and atherosclerotic lesion formation was reduced by 39%. Tijburg *et al.*, (1997) also found that drinking green tea for 21 weeks reduced atherosclerotic lesion formation in rabbits by 31%, but this decrease did not reach statistical significance ($p= 0.11$). In addition to these animal intervention studies, Sasazuki *et al.*, (2000) found, in an epidemiological study using coronary arteriography, that men who drank 2-3 or 4 cups of green tea per day had 50% and 60% less coronary atherosclerosis than those who didn't drink green tea respectively.

In summary, the ability of green tea and its catechins to inhibit LDL oxidation and lower plasma cholesterol concentrations indicates that they may reduce the progression of atherosclerosis and this is supported in the studies described above. The combined evidence from the literature suggests that green tea and its catechins may be protective agents against heart disease.

1.13 Experimental Rationale

As discussed previously, an increase in the LDL receptor is one mechanism by which green tea and its catechins may exhibit their hypocholesterolaemic effects. To this effect, previous *in vitro* studies in our laboratory, including work by the present author during her honours studies (Bursill 1996), found direct evidence that green tea and its catechins can upregulate the LDL receptor. When freshly brewed green tea (10% w/w) was included in the media of cultured human hepatoma (HepG2) cells, there was an increase in LDL receptor binding activity and in the relative amounts of LDL receptor protein (Bursill 1996). An ethyl acetate extract from green tea, enriched in catechins, was also able to increase LDL receptor binding activity (Figure 1.10) and protein as well as the relative amounts of LDL receptor mRNA. This indicated that the effect of green tea on the LDL receptor occurred at the level of gene transcription (Bursill 1996, Pal *et al.*, 1999).

As the catechins are the main constituents in green tea and the ethyl acetate extract, it was hypothesised that they may be the ingredients that caused the increase in the LDL

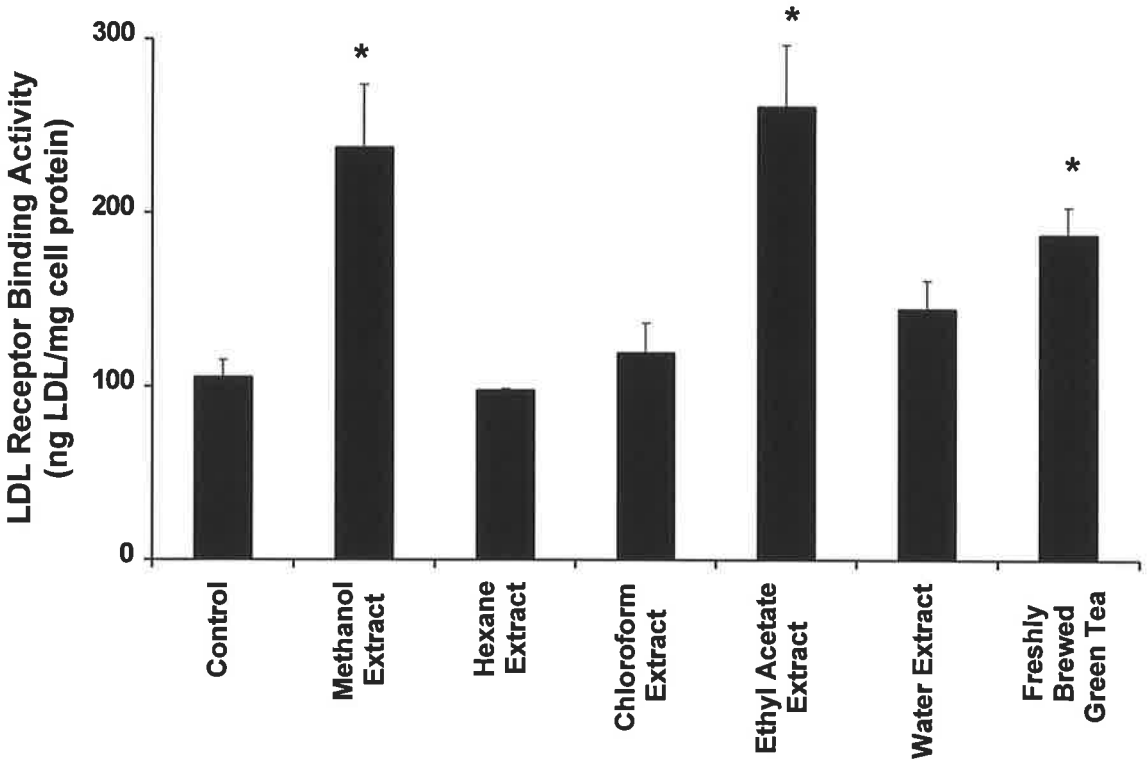


Figure 1.10. Effect of different green tea extracts on LDL receptor binding activity. HepG2 cells were incubated for 24 h with the indicated solvent extracts of green tea as prepared by the procedure illustrated in Figure 5.1. The concentration (w/v) of the ethyl acetate extract used in the media was calculated to represent 100 μM epigallocatechin gallate equivalence. For direct comparison, the same concentration (w/v) of the other extracts was used. The 10% (w/v) freshly brewed green tea (100 μl in 10 ml media) was used as a positive control. Values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to control ($p<0.05$).

receptor. Other studies in our laboratory also found that dietary antioxidants (Vitamin A, E, C and β-carotene) could increase the LDL receptor of HepG2 cells (Pal *et al.*, 1999). Since catechins are also potent antioxidants, perhaps the upregulation of the LDL receptor was an antioxidant effect. A comparative study, where purified samples (100μM) of the main catechins in green tea were included in the media of HepG2 cells, revealed that EGCG was the only catechin to significantly increase LDL receptor binding activity and the relative amounts of LDL receptor protein (Bursill 1996, Pal et al., 1999) (Figure 1.11). This indicated that EGCG was the active constituent in green tea that increased the LDL receptor.

Evidence that this effect of green tea and EGCG on the LDL receptor also occurred *in vivo* was found in rats (Bursill 1996) (Figure 1.12). The administration of a crude catechin extract from green tea to rats for 12 days significantly increased hepatic LDL receptor binding activity and the relative amounts of LDL receptor protein. Despite the increase in the LDL receptor there was no reduction in plasma lipids. This could not be explained by changes in either cholesterol absorption or cholesterol synthesis as they remained unchanged after treatment. This result did not support the other green tea studies in rats which found significant reductions in plasma lipids (Muramatsu *et al.*, 1986, Yang and Koo 1997, Yang and Koo 2000) despite supporting the increase in the LDL receptor found "in vitro" with HepG2 cells. Rats, however, have a strong ability to maintain their plasma cholesterol concentration (Fujioka *et al.*, 1995). Either hyper- or hypocholesterolaemia is not always seen in rats with cholesterol feeding or administration of cholesterol lowering drugs. A strong treatment effect is therefore required to produce changes and perhaps the 12 day treatment period was not sufficient. In contrast to the rat, the rabbit is extremely sensitive to changes in cholesterol

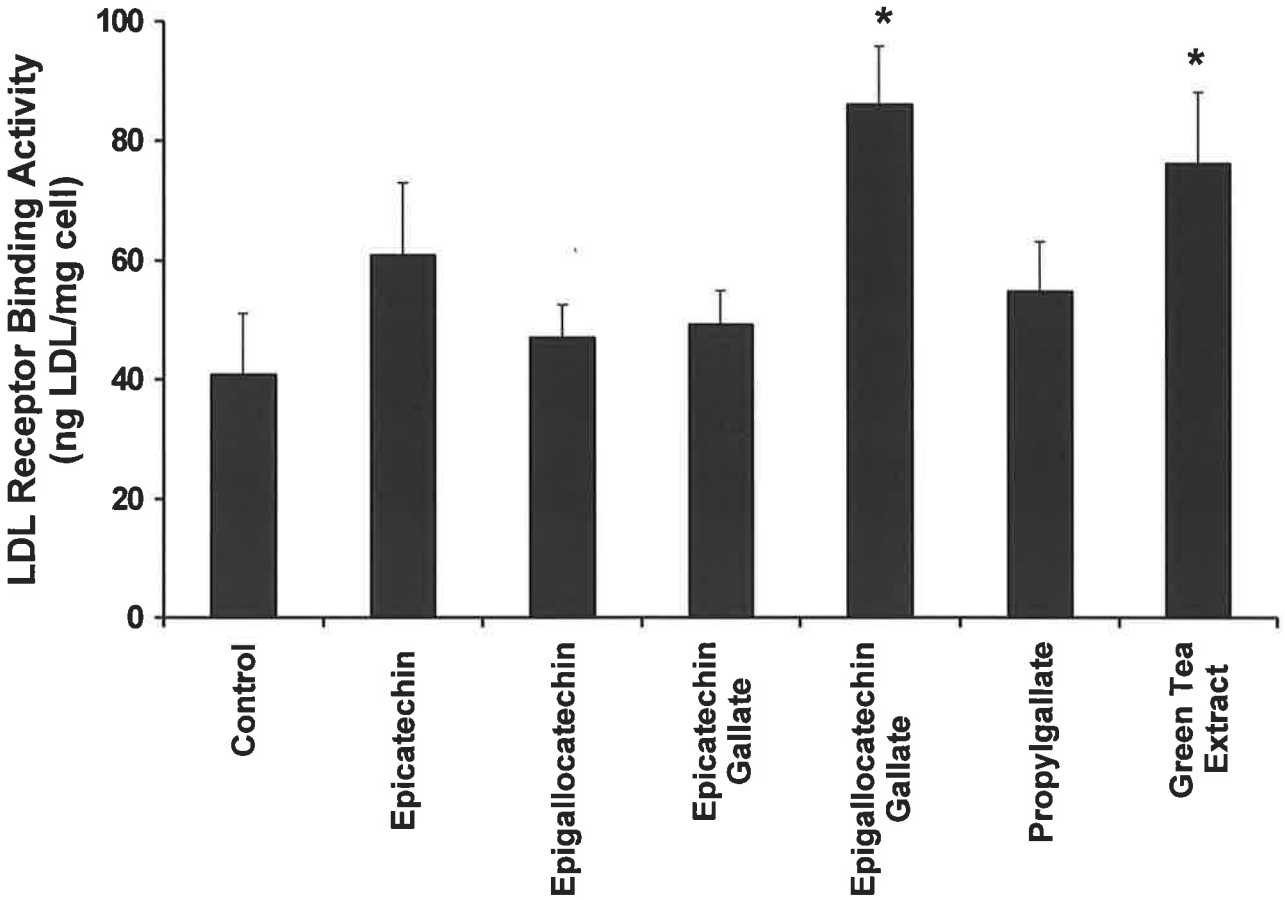


Figure 1.11. Comparison of purified catechins from green tea and a green tea extract on LDL receptor binding activity. HepG2 cells were incubated for 24 h with 100 μM of purified catechins or 100 μM EGCG equivalent of a green tea extract in 10 ml of media. The LDL receptor binding activity was measured as the calcium-dependent binding of LDL-gold to the intact cells. The values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to control ($p<0.05$).

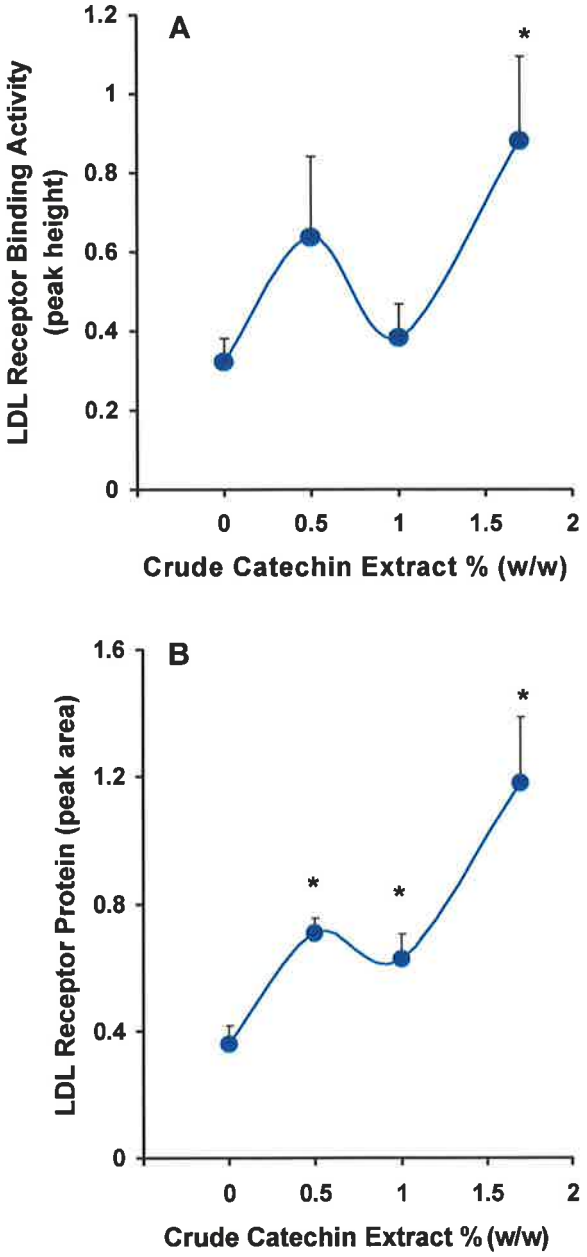


Figure 1.12. Effect of a crude catechin extract on the hepatic LDL receptor binding activity (A) and protein levels (B). Twenty four Sprague Dawley rats were divided into 4 different treatment groups and fed either 0, 0.5, 1.0 or 1.7% (w/w) of the crude catechin extract mixed in with normal rat chow along with 0.25% (w/w) cholesterol for 12 days. Hepatic LDL receptor activity was determined as the calcium-dependant binding of LDL-gold to solubilised liver membrane proteins blotted onto nitrocellulose. Relative amounts of LDL receptor protein were determined using a polyclonal antibody against the LDL receptor and western blotting. Values are expressed as means ± SEM. (*) denotes a significant difference compared to the control ($p < 0.05$).

administration with respect to plasma cholesterol concentrations and LDL receptor activity (Fujioka *et al.*, 1995). For the present study the rabbit was therefore thought to be a better animal model to use when studying the effects of green tea on cholesterol metabolism.

In summary, there is evidence *in vitro* and *in vivo* that green tea extracts and EGCG can increase the LDL receptor. This provides a possible mechanism to explain their hypocholesterolaemic effects in the epidemiological and animal intervention studies. The mechanism by which green tea and EGCG increase the LDL receptor is however, yet to be elucidated.

1.14 Overall Objectives

Green tea and its catechins have been found to exhibit hypocholesterolaemic properties. They have also been observed to upregulate the LDL receptor (Bursill 1996, Pal *et al.*, 1999), which is one possible mechanism by which green tea could lower plasma cholesterol, but the mechanism by which this occurs is unknown.

One aim of this research was therefore to determine the mechanism by which green tea and EGCG upregulate the LDL receptor in HepG2 cells. The LDL receptor can be regulated through changes in cellular cholesterol content, which modulates the level of the mature active form of sterol regulatory element binding proteins (SREBPs), transcription factors for the LDL receptor. These parameters were therefore investigated.

The other aim was to determine if a crude catechin extract from green tea could lower plasma cholesterol levels in the hypercholesterolaemic rabbit and ascertain if this effect is due to an increase in the LDL receptor. A reduction in cholesterol synthesis and cholesterol absorption from the intestine are other mechanisms by which a crude catechin extract could lower plasma cholesterol concentrations so these parameters of cholesterol metabolism were also investigated.

1.15 Research Protocols

To determine the mechanism by which freshly brewed green tea and EGCG increase the LDL receptor, these substances were included in the media of HepG2 cells and incubated for 24 h at 37 °C and 5% CO_2 (Chapters 4 and 5). LDL receptor binding activity was determined by the calcium-dependant binding of LDL-gold to the intact cells and LDL receptor protein was measured using western blotting. Intracellular total and unesterified cholesterol concentrations were measured on the homogenised cells. The conversion of the inactive precursor form of SREBP-1 to its mature active form was assessed using western blotting with a specific polyclonal antibody directed against the amino terminal of the SREBP-1. This antibody therefore allowed for the detection of both the inactive and active forms of SREBP-1. Bile acids, media cholesterol and cellular lathosterol (index cholesterol synthesis) concentrations were also determined using gas chromatography.

Studies on the effect of a green tea extract, enriched in catechins, on plasma lipids and the LDL receptor *in vivo* were performed in hypercholesterolaemic rabbits (Chapter 6). Twenty-four rabbits were initially made hypercholesterolaemic by feeding them 0.25% (w/w) cholesterol for a period of 2 weeks. After this, they were allocated into 4

different treatment groups based on body weight and plasma cholesterol concentrations and fed either 0, 0.5, 1 or 2% (w/w) of a green tea extract mixed in with their normal chow for 4 weeks. Following this diet regime rabbits were bled via cardiac puncture and their livers and aortas were excised. Lipid measurements were made on the plasma, livers and aortas and % fatty streak formation was assessed using lipophilic staining with oil red O. To determine the effect of the green tea extract on the hepatic LDL receptor, LDL receptor binding activity was measured by the binding of LDL-gold to solubilised liver membranes on nitrocellulose. The relative amounts of LDL receptor protein were also quantified using western blotting with a specific polyclonal antibody against the LDL receptor. Cholesterol synthesis and absorption were investigated using gas chromatography, measuring the plasma ratio of lathosterol to cholesterol and phytosterols to cholesterol respectively.

Chapter 2

Methods

2.0 METHODS

2.1 Cell Culture

2.1.1 Maintenance

Human hepatocarcinoma cells (HepG2 cells) were grown in continuous cell culture at 37°C under 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) with phenol red. DMEM was supplemented with 12 μg/ml penicillin, 16 μg/ml gentamycin, 20 mM HEPES, 2 mM glutamine and 10% (v/v) fetal calf serum (FCS) (TRACE Biosciences Pty Ltd, Australia). The FCS, antibiotics and glutamine were added to the media just before use (complete DMEM). Once made up the complete media could be stored at 4°C for 2 weeks. The cells were grown in 75 cm^2 flasks (Cellstar, Griener Labortechnik, Germany) and the media was changed every 2-3 days.

2.1.2 Growing of Cells for Experiments

When HepG2 cells reached confluence they were split into 2 or 3 new flasks depending on experimental requirements. The confluent cells were removed from flasks using 0.1% (w/w) trypsin-EDTA treatment. Initially, the culture media was removed and washed once with phosphate buffered saline (136 mM NaCl, 10 mM Na_2PO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, pH 7.4). Approximately 20 ml more of PBS was then added to the flask and left to sit for 2 min. This is done to remove all traces of free salts (e.g. Ca^{2+} and Mg^{2+}) from the cells. After 2 min the PBS was emptied from the flask and 5 ml of 0.1% (w/w) trypsin-EDTA was added to the flask and left to incubate for 5min. When trypsinisation was complete approximately 3 ml of complete DMEM was added to the cells and they were resuspended thoroughly with an automatic pipette. This was essential to ensure the cells grew in a monolayer. Cells

were then diluted with either 20 or 30 ml of complete DMEM and distributed evenly into 2 or 3, 75 cm^2 flasks. They were then grown without disturbance for at least 24 h in order to allow attachment.

2.2 Test for Normal LDL Receptor Function Before Experimental Intervention.

2.2.1 Preparation of Lipoprotein Deficient-Fetal Calf Serum (LPD-FCS).

Lipoproteins from FCS were removed by ultracentrifugation. FCS (initial density 1.006 g/ml) was adjusted to a final density of 1.215 g/ml with potassium bromide and centrifuged for 40 h (10°C) at 38, 000 rpm in a 50 Ti Beckman rotor. The floating layer of lipoproteins was removed and the bottom fraction was collected and dialysed at 4°C against 150 mM NaCl (5 changes of 2 litres each) for 72 h. After dialysis the LPD-FCS was sterilised by passage through a 0.45 μm Millipore filter, alliquoted into sterile 10 ml tubes and kept frozen at -20°C until required.

LPD-FCS culture media was prepared as 10% LPD-FCS in complete DMEM and stored at 4°C.

2.2.2 Incubation with LPD-FCS

Intracellular cholesterol concentrations and cholesterol concentrations in the media regulate the LDL receptor. When the cholesterol levels in the media are higher the LDL receptor is downregulated to reduce the influx of cholesterol into the cells and vice versa for lower media cholesterol concentrations (Brown and Goldstein, 1986). This criterion provides the basis in which to verify normal LDL receptor regulation before proceeding with subsequent experiments.

Cells were incubated for 24 h with DMEM media supplemented with either LPD-FCS (low cholesterol) or normal FCS + 100 µl isolated LDL (high cholesterol). At the end of the treatment period the cells were harvested and the relative amounts of LDL receptor protein were determined by methods outlined in Section 2.5. It was found that levels of LDL receptor protein were higher in the low cholesterol treated cells than the high cholesterol treated cells, indicating that the cells responded to normal LDL receptor regulation.

2.3 Treatment with Green Tea and EGCG

Green tea was prepared fresh on the day of experimentation. The green tea was commercially available "Special Gunpowder" packaged by the China National Native Products and Animal By-products Import and Export Corporation, Zhejiang Tea Branch, China. A 10 % (w/v) concentration of green tea was prepared in just-boiled water. This was brewed for 10 mins, filtered with filter paper then cooled. Purified EGCG was purchased from Sigma (Castle Hill, NSW, Australia). It was estimated to be 95% EGCG according to the manufacture's description. The green tea and EGCG were used in separate experiments and added to 10 ml of complete DMEM media in a concentration range of 0-200 µl and 0-200 µM respectively. This was then left to incubate for 24 h. Following incubation, the media was decanted, the cells were washed once with PBS and then a further 2 ml of PBS was added to the flask. The cells were harvested, by gentle scraping with a rubber scraper. For measurement of LDL receptor binding activity the intact cells were used immediately after harvesting. Once used, the remaining cells were frozen away at -80°C for at least 24 h to measure the relative amounts of LDL receptor protein.

2.4 Measurement of LDL Receptor Binding Activity in HepG2 Cells

2.4.1 Preparation of Colloidal Gold-LDL

Colloidal gold was prepared by the method of Frens (1973). A 500 ml solution of 0.01% (w/v) chloroauric acid was brought to boiling under reflux in a round bottomed flask which had been cleaned by overnight submergence in a sulfochromic acid bath and siliconised with 5% (v/v) dichloromethylsilane in carbontetrachloride (30 min). While boiling, 14 mL of 1% trisodium-citrate (w/v) solution was added. When the solution became red it was then boiled for a further 10 min to make sure the reaction was complete. The colloidal gold solution was stored at 4°C and used for up to two weeks. However, if stored under sterile conditions it is infinitely sterile.

Normolipidemic human blood (Australian Red Cross, Adelaide, Australia) was used to isolate LDL (1.025>d>1.063 g/ml) by sequential ultracentrifugation (Havel, 1955). LDL was then dialysed overnight against 154 mM NaCl and 1 mM EDTA at pH 7.5. The protein content of LDL was determined by the method of Lowry *et al.* (1951).

Isolated human LDL was conjugated to colloidal gold as described previously (Roach *et al.*, 1987). LDL was diluted to 1 mg/ml and dialysed overnight against 50 mM EDTA (pH 8.0). 150 μg (protein) of LDL was diluted with 0.5 ml of deionised water in 10 ml plastic centrifuge tubes. Colloidal gold solution (5 ml per tube) was then added rapidly while vortexing. The conjugates were pelleted at 20,000 x *g* for 20 min at 10°C. Tubes were left to stand until no more conjugates came off the side of the tubes. The supernatant was then aspirated off and the conjugates were collected. For

storage, the conjugates were mixed with 20% (w/v) sucrose and their protein content determined (Lowry *et al.*, 1951). This was aliquoted (1 ml) into 1.5 ml eppendorf tubes and could be kept for up to 6 months at -80°C. When required, the conjugates were thawed quickly in warm water. LDL-gold standards (12.5, 25, 50, 100 and 200 ng) were also prepared for the measurement of LDL receptor binding activity in HepG2 cells. The LDL-gold standards were prepared in 4% (w/v) gum arabic and stored in 120 µl aliquots at -80°C. The 4% (w/v) gum arabic solution was prepared by the method of Danscher (1981).

2.4.2 LDL Receptor Binding Activity in HepG2 Cells

Cellular LDL receptor binding activity was measured by the method of Roach *et al.* (1993). After treatment, the cells were harvested, pelleted and resuspended in PBS. The cellular protein was then determined (Lowry *et al.*, 1951). The intact cells (100 µg) were incubated in a total assay volume of 300 µl for 1 h at room temperature with LDL-gold conjugates (20 µg protein/ml) and 75 µl of incubation buffer (240 mM Tris-HCl, 100 mM NaCl, 8 mM $CaCl_2$, and 8% (w/v) bovine serum albumin (BSA), pH 8.0) in the absence or presence of 20 mM EDTA (pH 8.0) for total and non-specific binding respectively. After 1 h, the cells were pelleted by centrifugation at 400 x *g* and washed in 500 µl of either 2 mM $Ca(NO_3)_2$ for total binding or 20 mM EDTA (pH 8.0) for non-specific binding. Cells were again pelleted by centrifugation at 400 x *g* and resuspended in 120 µl of 4% (w/v) gum arabic. A silver enhancement solution (Amersham IntenSE BL kit, Sydney, Australia) was then added using the Cobas Bio autoanalyser (Roche Diagnostica, Nutley, NJ) and the absorbance measured at 500 nm after incubation for 33 min at 37°C. LDL-gold standards were included in each cobas run. Using the standard curve, the amount of LDL bound to

the cells was expressed as ng LDL protein bound per mg cell protein (ng LDL/mg cell). Duplicate determinations were made for both total and non-specific binding. Specific binding (total binding minus non-specific binding) was taken to be the measure of LDL receptor binding activity.

2.5 Measurement of LDL Receptor Protein in HepG2 Cells

2.5.1 Solubilisation of Cells

Prior to solubilisation, the cells were frozen at -80°C for at least 24 h after harvesting and then slowly thawed when required for experimentation. Thawed cells were pelleted by centrifugation at 400 x *g* for 10 min and resuspended vigorously in approximately 200-225 µl of solubilisation buffer (250 mM Tris-Maleate, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM N-ethylmaleimide, pH 6.0) to make a total volume of 300 µl in 1.5 ml eppendorf tubes. To this, 90 µl of 5% (v/v) Triton X-100 was added to the tubes and vortexed. Any unsolubilised cell matter was removed by pelleting using centrifugation at 400 x *g* for 15 min in a swinging bucket centrifuge and the protein content of the solubilised cell supernatant was determined (Lowry *et al.*, 1951). The solubilised cell samples (100 µg) were then combined with 10% (w/v) SDS and glycerol in a ratio of 6:3:1 (cells : 10% SDS : glycerol) to make up a volume no more than 200 µl, preferably less. Tracking dye (1 µl, 0.5% bromophenol blue) was then added to this mixture and vortexed thoroughly.

2.5.2 Separation of Cellular Protein

The proteins in the solubilised cell samples were separated on 3-15% SDS polyacrylamide gradient gels (Laemmli, 1970). RAINBOW molecular weight-

markers (Pharmacia LKB, Uppsala, Sweden) were also run along with the cell samples. In their preparation, 12 µl of the markers, 4.5 µl of 10% SDS, 9 µl 0.5 M Tris-HCl (pH 6.8), 2.7 µl glycerol and 1.6 µl β-mercaptoethanol (enough for two wells) were combined and then heated at 95 °C for 2 min. The samples were loaded into the wells and run at 60 mA until the dye front had run off the end of the gel into the running buffer (3 g/l Tris base, 14.4 glycine, 1 g/l SDS, pH 8.3). The gel was then removed from its plates and incubated in transfer buffer (25 mM Tris base, 192 mM glycine and 20% w/v methanol) for 30 mins. After this incubation, the separated proteins were electro-transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a transblot apparatus (Biorad, NSW, Australia) at 45V for 15 h at 4°C (Burnette, 1981). The LDL receptor was then detected by immunoblotting.

2.5.3 Immunoblotting

The nitrocellulose membranes were blocked by incubation on a platform rocker with 10% (w/w) skim milk powder (SMP) buffer (10 mM Tris-HCl, 154 mM NaCl, pH 7.4) for 1 h. They were then washed 2 x 10 min with 1% (w/w) SMP in a 1:1 dilution of the buffer (buffer : water) just described. This was followed by a 1 h incubation with a polyclonal antibody directed against the LDL receptor (Roach *et al.*, 1993) diluted 1:2000 in 1% (w/w) SMP buffer. After washing for another 3 x 10 min the nitrocellulose membranes were incubated for a further 1 h with a second antibody conjugated to horse radish peroxidase (Amersham, UK) diluted 1:5000 with 1% (w/w) SMP buffer. At the completion of this incubation the membranes were washed 2 x 5 mins in 10 mM Tris-HCl, 154 mM NaCl and 2 mM $CaCl_2$ (pH 7.4). The LDL receptor band was then decteted on X-ray film (Hyperfilm-ECL, Amersham, North

Ryde, NSW, Australia) using an enhanced chemiluminescence kit from Amersham (Krika, 1991). Quantification of the LDL receptor protein on film was performed using an LKB Ultroscan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, NSW, Australia). Results were expressed as peak area, determined from the laser densitometer scan.

2.6 Measurement of Hepatic LDL Receptor Binding Activity and Protein in Rabbits

Rabbit livers were homogenised and solubilised as described in Section 5.2.5.1. Hepatic LDL receptor binding activity was determined by measuring the calcium-dependant binding of LDL-gold to solubilised liver membranes dot blotted onto nitrocellulose (Section 5.2.5.2). Relative amounts of hepatic LDL receptor protein were determined using the solubilised liver membranes (100 μg), with a polyclonal antibody against the LDL receptor and western blotting (Section 2.5).

2.7 Measurement of SRE-BP 1c

2.7.1 Cell Culture

HepG2 cells were treated in the same manner as described in section 2.3 with one exception; 16 h prior to harvesting all cells received 25 μg/ml of N-acetyl-leucine-leucine-norleucinal (N-ALLN). Five 75cm^2 flasks were required for each treatment group in order to obtain sufficient amounts of protein. After the treatment period the cells were harvested into sterile 50 ml Falcon tubes and spun down at 500 x *g* for 10 min.

2.7.2 Preparation of Nuclear and Membrane Fractions

The cells were fractionated by the method described by Wang *et al.* (1994). The pelleted cells were resuspended in 5 volumes (approximately 3 ml) of homogenisation buffer (10 mM HEPES-KOH at pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM sodium EDTA, 1 mM sodium EGTA) which was supplemented with proteinase inhibitors (0.1 mM pefabloc, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, 2 μg/ml aprotinin). The cells were then disrupted by passage through a 22 gauge needle 15 times and the resulting homogenate was centrifuged at 1000 x *g* for 10 min. The resulting crude nuclear pellet was then resuspended with an equal volume (approximately 1 ml) of solubilisation buffer (20 mM HEPES-KOH at pH 7.6, 25% v/v glycerol, 0.5 M NaCl, 1.5 mM MgCl2, 1mM EDTA, 1 mM EGTA) supplemented with proteinase inhibitors and centrifuged at 15, 000 rpm in a Beckman TLA 120.2 rotor in an Optimal TLX benchtop ultracentrifuge (Beckman Instruments, CA, USA) for 30 min. The supernatant from this spin was designated the nuclear extract and its protein content was determined (Lowry *et al.*, 1951).

The supernatant from the initial low speed spin (1000 x *g*) was spun at 100, 000 x *g* for 1 hr to obtain the membrane fraction (pellet). This was then resuspended in 160 μl of 1% (w/v) SDS in buffer (10 mM Tris-HCl [pH 6.8], 100 mM NaCl) and incubated at room temperature for 15 min. The protein content was then determined (Lowry *et al.*, 1951).

2.7.3 Immunoblotting

Aliquots (150 μg) of the nuclear and membrane fractions were added to 2 volumes of loading buffer (Laemmli 1970) and boiled for 3 min. Once cooled, samples were

loaded onto an 8% SDS PAGE gel (Laemmli 1970) and run at 100 mA for approximately 90 mins (4°C). Protein was then transferred at 20 V overnight at 4°C onto nitrocellulose membranes. The membranes were then overlayed with a polyclonal antibody against the SREBP 1c protein (1:200 dilution, Santa Cruz, CA, USA) followed by a secondary antibody conjugated to horseradish peroxidase (1:5000 dilution). The inactive precursor and the active mature transcription factor forms of SREBP-1c were then detected on X-ray film (Hyperfilm-ECL, Amersham, North Ryde, NSW, Australia) using enhanced chemiluminescence (SuperSignal Substrate, Rockford IL, USA). Quantification of the inactive precursor and the active mature transcription factor forms of SREBP-1c were performed using laser densitometry (LKB Ultroscan XL enhanced laser densitometer, Pharmacia LKB Biotechnology, North Ryde, NSW, Australia).

2.8 Total Cholesterol, Unesterified Cholesterol and Cholesterol Synthesis Assays.

2.8.1 Preparation of Cells

Total cholesterol, unesterified cholesterol and lathosterol (an index of cholesterol synthesis, Kempen *et al.*, 1988) were all measured in homogenised HepG2 cells after treatment with either green tea or EGCG. In preparation for these measures, treated cells were frozen at -80°C for at least 24 h and slowly thawed when required for experimentation. Thawed cells were subjected to centrifugation for 10 min at 400 x *g*. They were then homogenised in 1 ml of SDS buffer (0.1% w/v SDS, 1 mM EDTA and 0.1 M Tris Base, pH 7.4) by passage through an 18 gauge needle 4-8 times. Protein content was then determined (Lowry *et al.*, 1951).

2.8.2 Preparation of Media

Total cholesterol concentration in the media of HepG2 cells was also measured after treatment with either green tea of EGCG. Media (10 ml) was reduced down to near dryness using a Savant SpeedVac SC100 (Selby Anax, Adelaide, Australia) then resuspended in 1 ml of water ready for anaylsis.

2.8.3 Measurement of Total Cholesterol, Unesterified Cholesterol and Lathosterol

Total cholesterol, free cholesterol and lathosterol were determined in cells and/or the media of the cells using gas chromatography as described by Wolthers *et al.* (1991). Standard solutions of sterols were prepared in hexane in the concentration ranges 5-400 μg/ml for cholesterol and 2.5-200 μg/ml for lathosterol. Two hundred μl of standards were added to kimble tubes containing 30 μl of internal standard (1 mg/ml 5β-cholestan-3α-ol), dried under a stream of nitrogen and reconstituted in 200 μl of water. For preparation of cell and media samples, 30 μl of internal standard was dried down the bottom of a kimble tube and then the pre-prepared cell and media samples were added to these tubes. The standards and samples were hydrolysed by the addition of 100 μl of 33% (w/v) potassium hydroxide and 2 ml of ethanol. This was incubated for 30 min in a water bath set at 60°C. For the measurement of unesterified cholesterol this hydrolysis step was omitted. Sterols were extracted by the addition of 1 ml of distilled water and 2 ml of hexane then vortexing for 2 min. The upper hexane layer was collected and evaporated to dryness under nitrogen. Samples were then derivatised by incubation with 100 μl of Trisil-TBT (Power Sil-Prep Kit, Alltech, Deerfiled, IL) for 30 min at 80°C. Liquid extraction was performed with 4 ml of hexane, 4 ml of 0.1 M HCl and vortexing. The hexane layer was collected and washed with 4 ml of water. The upper hexane layer was then transferred into a reacti vial,

dried down with nitrogen and then redissolved in a further 50 µl hexane for injection onto a gas chromatograph.

2.8.4 Gas Chromatograph Conditions

The gas chromatograph (GC) used was a DANI 6500 with a split/splitless injection system (split ratio 1:20) set at a temperature of 250°C and a vitrous silica column (25 cm x 0.25 mm, 1 mm film thickness). The carrier gas was hydrogen. The retention times of the sterols were 8.8 min for the internal standard, 10.5 min for cholesterol and 11.6 min for lathosterol.

2.9 Measurement of Bile Acids

Cells were grown to near confluency in 75 cm^2 flasks as described in Section 2.3 except that the incubations with green tea and EGCG (24 h) were done in DMEM media that was free of phenol red (Axelson *et al.*, 1991). After the 24 h incubation, the media from two flasks was combined and the bile acids were extracted from the 20 ml of media by passage through a reverse phase C18 cartridge (Waters Associates, Milford, MA). This was washed with 10 ml of water and 5 ml of 10% (v/v) methanol. The bile acids were eluted with 85% (v/v) methanol into a 10 ml kimble tube and dried under a stream of nitrogen (Axelson *et al.*, 1991). Methylation was performed by incubation with a few drops of HCL in methanol for 2 h and then dried. The samples were derivatised by adding 100 µl of trifluroacetic anhydride and heated at 30 °C for 60 min, then dried (Ross *et al.*, 1977). The samples were reconstituted in 1 ml of hexane, vortexed for 2 min, transferred to a reacti vial, dried and resuspended in a further 50 µl of hexane. Approximately 3 µl was loaded on to the gas

chromatograph for analysis. Due to the low concentration of bile acids in Hep G2 cell media only chenodeoxycholic acid was successfully detected. This was quantified using a standard curve and calculated with respect to the internal standard (lithocholic acid).

The GC conditions were the same as described in section 2.8.4. The retention times were 10.6 min for chenodeoxycholic acid and 12 min for lithocholic acid.

Chapter 3

FRESHLY BREWED GREEN TEA MODULATES CHOLESTEROL METABOLISM IN CULTURED HUMAN LIVER (HEPG2) CELLS.

3.1 Introduction

Elevated plasma cholesterol is a major risk factor for the development of heart disease (Assman *et al.*, 1999). Green tea is a widey consumed beverage brewed from the plant species 'Camellia sinensis (L.) O. Kuntze' and has been found to exhibit hypocholesterolaemic effects. Epidemiological studies (Kono *et al.*, 1992, Imaj *et al.*, 1995, Kono *et al.*, 1996) have found that drinking between 5-10 cups of green tea per day is associated with lower plasma cholesterol concentrations (See Section 1.12.2). Green tea drinking has also been found to be inversely related to LDL cholesterol concentrations (Imaj *et al.*, 1995, Kono *et al.*, 1996) and positively related to HDL cholesterol (Imaj *et al.*, 1995). An epidemiological study by Sasazuki *et al.*, (2000) discovered, using arteriography, that people who drank 2-3 and 4 or more cups of green tea per day had 50% and 60% lower coronary atherosclerosis respectively compared to people who did not drink green tea.

Intervention studies in rats, mice and hamsters have also found that green tea or green tea extracts enriched in catechins exhibit hypocholesterolaemic effects (Muramatsu *et al.*, 1986, Matsuda *et al.*, 1986, Fukuyo *et al.*, 1986, Yang and Koo 1997, Chan *et al.*, 1999 and Yang and Koo 2000) (Section 1.12.2). In these studies, the administration of various green tea extracts enriched in catechins for 4-8 weeks significantly lowered plasma cholesterol concentrations by 20-40%. The most dramatic changes in plasma lipids occurred in a study by Fukuyo *et al.*, (1986) where 1% (w/w) of pure EGCG was administered to rats for a period of 4 weeks. This treatment lowered plasma and LDL cholesterol by 50% and 68% respectively. This last study indicates that perhaps EGCG alone has more potent hypocholesterolaemic effects than green tea or the other

catechins combined. In contrast to these findings, Tijburg *et al.* (1997) found that a green tea extract, included in the drinking water, did not significantly decrease cholesterol concentrations in the cholesterol-fed hypercholesterolaemic rabbit.

Studies in our laboratory (Bursill 1996, Pal *et al.*, 1999) have found that the addition of freshly brewed green tea to the media of cultured human liver (HepG2) cells significantly increased LDL receptor binding activity and the relative amounts of LDL receptor protein and mRNA. The hypocholesterolaemic effects of green tea may therefore be due to an increase in the low-density lipoprotein (LDL) receptor, a cell surface protein, which is the main mechanism by which cholesterol can be removed from the circulation (Brown and Goldstein 1986). The mechanism by which green tea increases the LDL receptor has not, however, been investigated.

The aim of the present study was therefore to determine the mechanism by which green tea upregulated the LDL receptor. The LDL receptor can be regulated through changes in cellular cholesterol content, which in turn modulates the level of the mature active form of sterol regulatory element binding proteins (SREBPs), transcription factors for the LDL receptor (Brown and Goldstein 1998). These parameters were therefore investigated. For this purpose, human HepG2 liver cells, known to express LDL receptors amenable to regulation (Havekes *et al.*, 1987), were cultured in the presence of increasing amounts of green tea.

3.2 Materials and Methods

3.2.1. Green Tea

The green tea used in this study was commercially available "Special Gunpowder" packaged by the China National Native Products and Animal By-products Import and Export Corporation, Zhejiang Tea Branch, China. The green tea was prepared fresh for every experiment by brewing 10 g of green tea leaves for 10 min in 100 ml of just-boiled hot water (10% w/v) followed by paper filtration.

3.2.2. HepG2 Cell Culture

The HepG2 cells were grown in monolayer cultures to near confluency in 75 cm^2 flasks with 10 ml of Dulbecco's modified Eagle's media (DMEM) containing 10% (v/v) fetal calf serum (DMEM/FCS) at 37°C with 5% CO_2 as described in Section 2.1. The cells were then incubated for 24 h in 75 cm^2 flasks with 10 ml of DMEM/FCS containing different amounts (0-200 µl) of the 10% (w/v) freshly brewed green tea (Section 2.3).

3.2.3 LDL Receptor Binding Activity

Following incubation, the cells from each flask were harvested, resuspended in phosphate buffered saline (PBS) and the protein content was determined (Lowry *et al.*, 1951). Determination of the specific LDL receptor binding activity was measured as described in Section 2.4.

3.2.4 SREBP-1c

Five 75cm^2 flasks/treatment group were harvested and the cells from these 5 flasks were pooled. Cells were fractionated and the relative amounts of both the inactive precursor and active transcription factor forms of SREBP-1c protein were determined as described in Section 2.7

3.2.5 Cholesterol, Lathosterol and Chenodeoxycholic Acid.

Cells were frozen at -80°C for at least 24 h and slowly thawed for analysis. Thawed cells were pelleted by centrifugation for 5 min at 400 x *g*. They were then homogenised and protein content determined (Lowry *et al.*, 1951). Total cholesterol (esterified plus unesterified), unesterified cholesterol and lathosterol (index of cholesterol synthesis) were then measured on the homogenised cells as described in Section 2.8. The cholesterol and lathosterol concentrations were expressed relative to the amount of cell protein (mg/mg cell protein and μg/mg cell protein respectively).

The cholesterol and chenodeoxycholic acid concentrations in the media were also determined. For cholesterol, 10 ml of the media was reduced down to near dryness using a Savant SpeedVac SC100 (Selby Anax, Adelaide, Australia), resuspended in 1 ml of water and analysed as for the cells (Section 2.8). For chenodeoxycholic acid, the cells were grown to near confluency in 75 cm^2 flasks as described above except that the green tea incubations (24 h) were done in 10 ml of DMEM media free of phenol red (Axelson *et al.*, 1991). After the 24 h incubation, the media from 2 flasks was combined (20 ml/sample) and chenodeoxycholic acid was quantified as described in Section 2.9.

3.2.6 Statistical Analysis

Results are expressed as mean ± SEM. Statistical evaluation was done using either a linear regression (SPSS software), a one-way ANOVA with Fishers least significant difference (LSD) or Bonferroni post hoc test of significance or a two-tailed Student t-test, comparing the control with the different treatment groups where appropriate. A value of $p<0.05$ was the criterion of significance.

3.3 Results

3.3.1 Green Tea and the LDL Receptor

As shown previously (Bursill 1996, Pal *et al.*, 1999), the addition of increasing amounts of freshly brewed green tea to the media of the HepG2 cells increased LDL receptor binding activity compared to the control. This effect of green tea occurred in a dose-dependent and saturable fashion (Figure 3.1A). Due to this saturation effect there was no significant linear trend between LDL receptor binding activity and the dose of green tea. There was, however a positive log linear response between LDL receptor binding activity and the log of the dose of green tea ($r^2 = 0.936$, $p< 0.01$) (Figure 3.1B). The amount of LDL-gold binding to the intact HepG2 cells was found to be significantly greater (+35%) than the control ($p < 0.05$) with the addition of only 10 µl of the tea to 10 ml of the media. It then attained a plateau from 50 µl onward to a maximum significant increase of 83% above the control levels at 200 µl ($p < 0.05$).

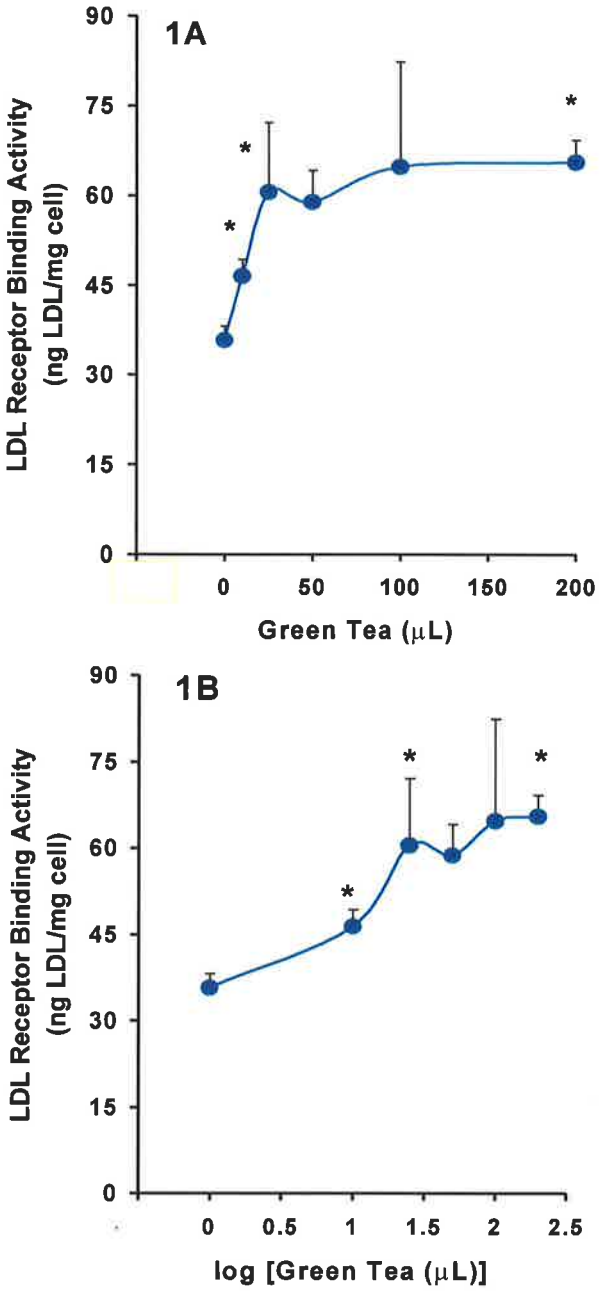


Figure 3.1. Dose-dependent effect of freshly brewed green tea on the LDL receptor binding activity (1A). 1B represents the log transformation of the dose of green tea in relation to LDL receptor binding activity. HepG2 cells were incubated for 24 h with increasing amounts of freshly brewed green tea, 0-200 µl in 10 ml of media. The LDL receptor binding activity was measured as the calcium-dependent binding of LDL-gold to the intact cells. The values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers LSD ($p< 0.05$).

3.3.2 Green Tea and Cell Cholesterol

There was no significant linear trend between cellular total cholesterol concentrations (esterified plus unesterified cholesterol) and the dose of green tea. This is because cellular total cholesterol concentrations were significantly decreased at each of the doses tested with the majority of the decrease occurring (-30%) at the lowest dose of 50 µl in 10 ml of media and then the effect attained a plateau ($p < 0.05$) (Figure 3.2). There was, however, a significant inverse linear trend between intracellular unesterified cholesterol concentration and the dose of freshly brewed green tea incubated with the cells ($r^2 = 0.953$, $p < 0.01$). This led to a significant decrease in the intracellular concentration of unesterified cholesterol (-25%) in the highest dose group of 200 µl green tea compared to the control (Figure 3.2).

3.3.3 Green Tea and SREBP-1c

Treatment with green tea (200 µl) resulted in a +62% (150 µg cell protein) and +65% (200 µg cell protein) increase in the active transcription factor form of SREBP-1c (nuclear cell fraction {N}, lanes 5 and 6), compared to the respective control (lanes 1 and 2) (Figure 3.3). In addition to this, green tea treatment decreased the inactive precursor form of SREBP-1c (membrane fraction; M) to undetectable levels (lanes 7 and 8 vs lanes 3 and 4).

3.3.4 Green Tea, Cholesterol Synthesis, Media Cholesterol and Chenodeoxycholic Acid.

The cellular lathosterol concentration, measured as an index of cholesterol synthesis (Wolthers *et al.*, 1991 and Kempen *et al.*, 1988), revealed that green tea significantly reduced cholesterol synthesis (-33%) at the lowest dose of 50 µl (Figure 3.4A). There

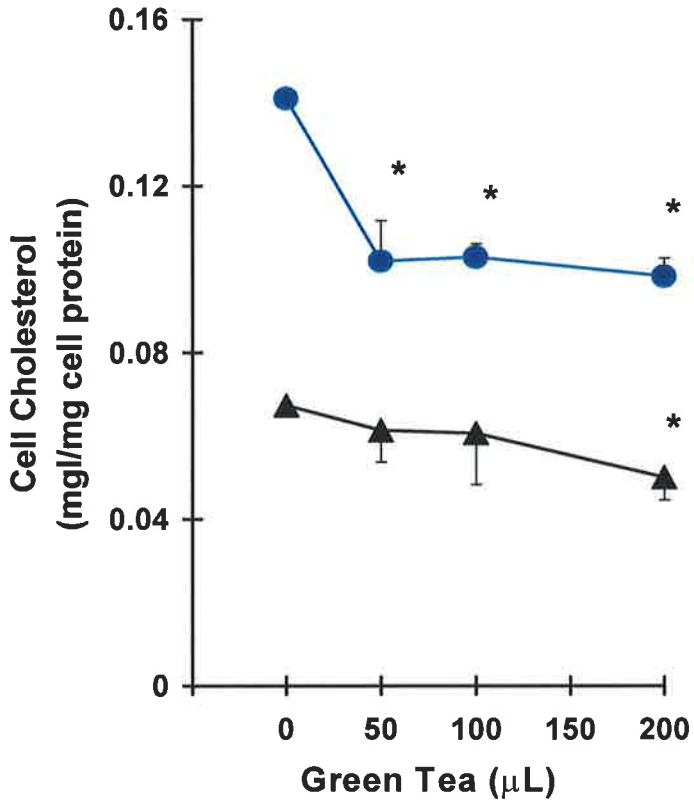


Figure 3.2. Dose-dependent effect of freshly brewed green tea on intracellular total and unesterified cholesterol concentrations. The HepG2 cells were incubated for 24 h with increasing amounts of green tea, 0-200 μl in 10 ml of media. Homogenised cells were extracted with hexane and total (●) and free (▲) cholesterol was analysed using gas chromatography and expressed relative to cell protein. The values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to control using a one way ANOVA with both the Bonferroni and Fishers LSD post hoc tests of significance ($p < 0.05$).

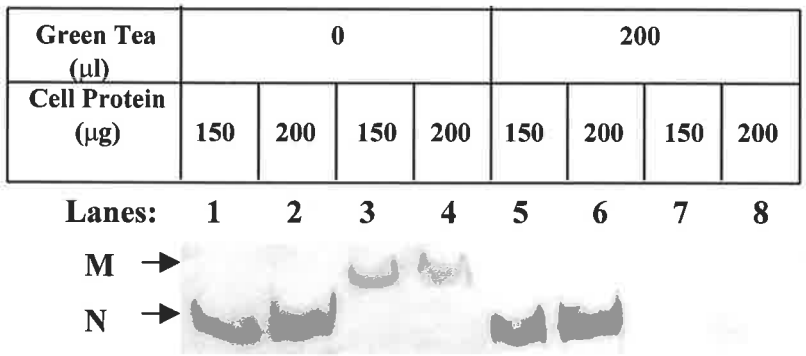


Figure 3.3. The effect of green tea on SREBP-1c. Cells were fractionated for nuclear and membrane fractions following incubation in the presence of freshly brewed green tea. Cellular proteins were separated and identified using SDS-PAGE and Western blotting (see methods). The inactive precursor (membrane form; **M**) and the active transcription factor form (nuclear cell fraction; **N**) of SREBP-1c were then detected on x-ray film using enhanced chemiluminescence. Lanes 1-4 represent cells that have not been exposed to green tea. Lanes 5-8 represent cells exposed to green tea.

was no significant difference from control at the higher dose of 100 µl, and at 200 µl, there was a significant 2-fold increase in the cellular lathosterol concentration (Figure 3.4A).

At the lower doses of 50 and 100 µl in 10 ml of media, the green tea tended to lower the cholesterol concentration in the media but this did not reach statistical significance (Figure 3.4B). At the highest dose of 200 µl however, green tea caused a significant increase (+25%) in the media cholesterol concentration (Figure 3.4B), indicating that there was an increased export of cholesterol from the cells to the media. This increased excretion of cholesterol into the media most likely caused the intracellular cholesterol to remain significantly decreased (Figure 3.2) despite an apparent increase in cholesterol synthesis at this dose (Figure 3.4A). There was a very high correlation ($r=0.956$, $p<0.01$) between the cell lathosterol concentration (Figure 3.4A) and the media cholesterol concentration (Figure 3.4B).

The green tea did not produce any significant changes in the concentration of chenodeoxycholic acid in the media (Figure 3.4B). The lowered intracellular cholesterol concentration (Figure 3.2) was therefore not likely to be due to an increase in the conversion of cholesterol to bile acids.

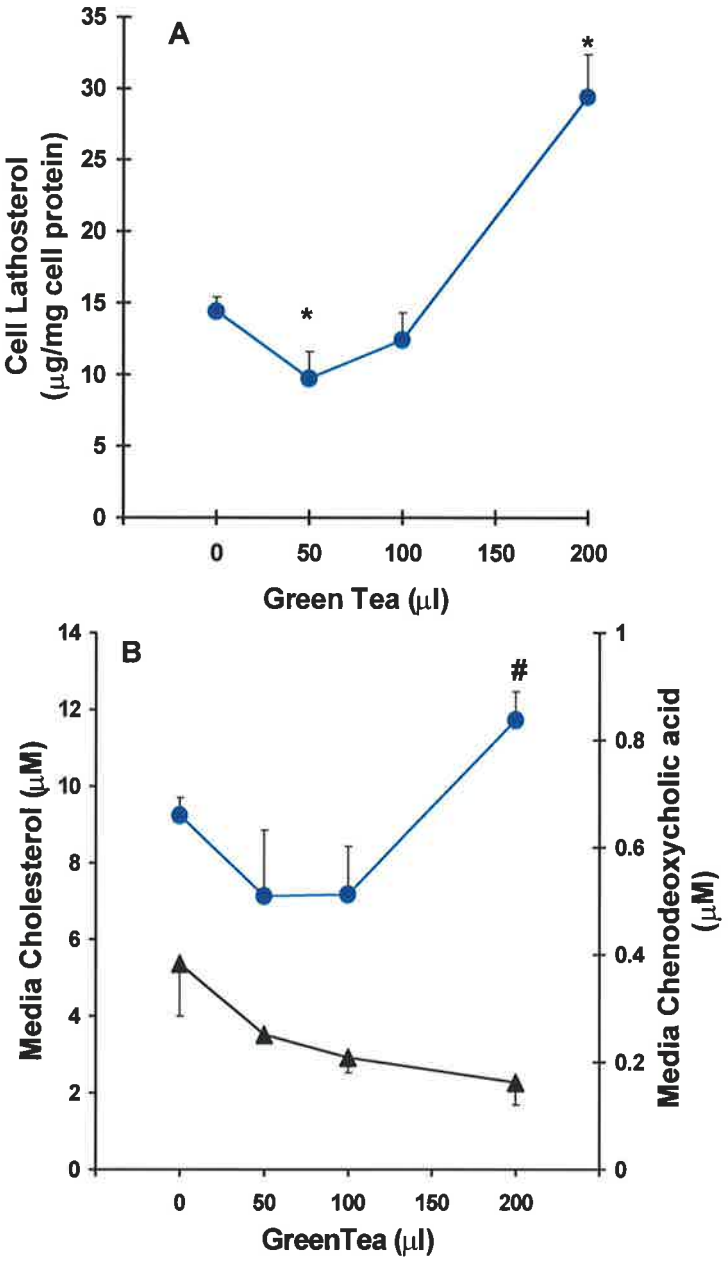


Figure 3.4. Dose dependant effect of freshly brewed green tea on cholesterol synthesis. HepG2 cells were incubated for 24 h with increasing amounts of green tea, 0-200µl in 10 ml media. Lathosterol (A,●) was extracted from homogenised cells and measured using gas chromatography. Media cholesterol (B, ●) and chenodeoxycholic acid (B, ▲) were also determined using gas chromatography. Values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to control using a one-way ANOVA with Fishers LSD post hoc test (p<0.05). The ($^{\#}$) denotes a significant difference compared to the control using a two-tailed t-test (p<0.05).

3.4 Discussion

The inclusion of freshly boiled green tea to the media of HepG2 cells increased LDL receptor binding activity. This is in agreement with previous studies (Bursill 1996, Pal *et al.*, 1999), which have found that freshly boiled green tea could also increase the relative amounts of LDL receptor protein and mRNA (Bursill 1996, Pal *et al.*, 1999). An increase in the LDL receptor may therefore explain the hypocholesterolaemic effect of green tea and green tea extracts that have been found in epidemiological and animal intervention studies.

The mechanism by which freshly brewed green tea increased the LDL receptor was then investigated. It was found that when hepatocytes were incubated in the presence of freshly brewed green tea, cell cholesterol was 30% lower than control. Whilst a lowering of cellular cholesterol can trigger an increase in the LDL receptor through a sterol negative feedback system (Brown and Goldstein 1986), it is the unesterified form of cholesterol which is thought to be regulatory (Brown and Goldstein 1998). This is because unesterified cholesterol can be converted to oxysterols (Grundy 1991) which regulate the activation of SREBP's by inhibiting their proteolytic cleavage from the endoplasmic reticulum. A decrease in unesterified cholesterol therefore reduces the formation of oxysterols and allows the activation of the SREBP's to their mature transcription factor form. Consistent with this, the present study found that green tea treatment lowered unesterified cholesterol concentrations by 25% and there was a significant inverse linear trend between the dose of green tea and unesterified cholesterol ($p<0.05$). Furthermore, incubation with 200 µl of green tea increased the conversion of SREBP-1c from its inactive precursor form to its active transcription

form. Green tea therefore appeared to cause a deficiency in cell cholesterol, which triggered the activation of SREBP-1, which in turn activated the LDL receptor gene to increase the production of LDL receptor protein. Taken together these findings provide a mechanism to explain the upregulation of the LDL receptor by green tea.

The decrease (-30%) in total cellular cholesterol caused by treatment with freshly boiled green tea could have occurred via three mechanisms: an inhibition of cholesterol synthesis, an increase in the efflux of cholesterol from the cell to the media and an increase in the conversion of cholesterol to bile acids. At the lowest dose of green tea (50 µl in 10 ml of media) there appeared to be a decrease in cholesterol synthesis as measured using cell lathosterol, a cholesterol precursor used as an index of cholesterol synthesis (Kempen *et al.*, 1988). At this concentration, green tea may have inhibited cholesterol synthesis which, in turn, may have contributed to the reduction in cell cholesterol. One possible mechanism by which green tea may have lowered cholesterol synthesis is to inhibit HMGCoA reductase, the rate-limiting enzyme in cholesterol synthesis but this was not investigated in this study (See Sections 5.4 and 6.1.1).

In contrast to the effect at the lowest dose of green tea (50 µl), cell lathosterol was increased 2-fold over control at the highest dose of green tea (200 µl). Despite this apparent increase in cholesterol synthesis, cholesterol did not accumulate within the cells, as the cell cholesterol remained decreased by more than 30%. Instead of accumulating in the cells, the extra cholesterol was found in the media where its concentration was increased by +25% over control. It therefore appears that at the highest concentration, green tea increased the export of cholesterol from the cells into the media to such an extent that the increase in cholesterol synthesis did not fully

compensate for the loss of cell sterol. Interestingly, there was a very high correlation (r = 0.956) between cell lathosterol and the concentration of cholesterol in the media, suggesting that the concentration of cholesterol in the media was directly linked to the amount of cholesterol synthesised by the cells. The increase seen in the SREBP-1c mature form with 200 µl of green tea is also consistent with the observed increase in cholesterol synthesis as SREBP-1c also upregulates the HMG-CoA reductase gene, the rate limiting enzyme in cholesterol synthesis.

Epigallocatechin gallate, the most abundant catechin in green tea, has been found to form insoluble complexes with cholesterol (Ikeda *et al.*, 1992). This could explain the effects seen *in vitro* with the highest dose of green tea in the present study. At this concentration the catechins may complex with enough cholesterol in the media to render the media essentially cholesterol-deficient. Cholesterol could then move from the cells into the media by normal diffusion down the concentration gradient. This could explain why the concentration of cholesterol is seen to increase in the media while the cells are not able to regain their normal intracellular cholesterol levels despite an increase in cholesterol synthesis.

An increase in the conversion of cell cholesterol into bile acids (Axelson *et al.*, 1991) did not appear to be a factor in the reduction of cell cholesterol. The chenodeoxycholic acid content of the media did not change significantly after treatment with green tea.

In conclusion, upregulation of the LDL receptor by freshly brewed green tea may be mediated through an increased activation of SREBP-1c in response to a decrease in cellular cholesterol concentration. Cellular cholesterol appears to be lowered by

inhibiting cholesterol synthesis at the lower doses (50 µl) and increasing the efflux of cholesterol from the cells to the media at the highest dose (200µM).

The EGCG is likely to be the active ingredient in green tea that modulates cholesterol metabolism in HepG2 cells. According to the literature EGCG exhibits the same effects on cholesterol metabolism as green tea. For example, both have been found to exhibit hypocholesterolaemic effects in animal models (Fukuyo *et al.*, 1986, Chisaka *et al.*, 1988). In fact, the greatest reduction in plasma lipids occurred with administration of pure EGCG (Fukuyo *et al.*, 1986) (Section 1.12.2). In our laboratory, EGCG has also been found to increase LDL receptor binding activity and protein in HepG2 cells (Bursill 1996, Pal *et al.*, 1999). This provided evidence that EGCG was the active ingredient in green tea which modulates cholesterol metabolism. This was further investigated in Chapter 4.

Chapter 4

EPIGALLOCATECHIN GALLATE (EGCG) MODULATES CHOLESTEROL METABOLISM IN CULTURED HUMAN LIVER (HEPG2) CELLS.

4.1 Introduction

The inclusion of freshly brewed green tea to the media of HepG2 cells has been found to modulate cholesterol metabolism in HepG2 cells (Chapter 3). This treatment lowered intracellular total and unesterified cholesterol and increased the conversion of SREBP-1c to its mature active form, providing a mechanism to explain the upregulation of the LDL receptor by green tea. It was also found that green tea produced a biphasic "down-then-up" effect on cholesterol synthesis and appeared to increase the efflux of cholesterol from the cells in the highest dose treatment group.

Green tea contains an abundance of polyphenolic compounds called catechins and there are four main types in green tea: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin gallate (ECG) and (-)-epigallocatechin gallate (EGCG). Catechins account for more than 30% of the solids in a normal infusion of green tea (Graham 1992, Harbowy and Balentine 1997) and of these, EGCG is the most abundant accounting for 58% of the total catechins (Muramatsu *et al.*, 1986). EGCG may therefore be the active constituent in green tea that produces the hypocholesterolaemic effects described. In support of this, studies have found that EGCG exhibits similar effects on cholesterol metabolism as green tea. For example, the administration of EGCG to mice (Matsuda *et al.*, 1986) and rats (Fukuyo *et al.*, 1986, Chisaka *et al.*, 1988) has also been observed to significantly lower plasma cholesterol concentrations just as well, if not more dramatically that green tea extracts. In addition to this, studies in our laboratory have found that the inclusion of EGCG to the media of HepG2 cells also effectively increased LDL receptor binding activity and protein (Bursill 1996, Pal *et al.*, 1999). In these *in vitro* studies, treatment with the other main catechins in green tea had no effect on the LDL receptor, suggesting that EGCG was the active ingredient in green tea that

increases the LDL receptor at least. If, therefore, EGCG is found to modulate cholesterol metabolism in the same fashion as freshly brewed green tea was found to in Chapter 3, it will provide further evidence that it is the active ingredient.

The aim of the present study was therefore to determine whether EGCG upregulated the LDL receptor in the same way as freshly brewed green tea. The LDL receptor can be regulated through changes in cellular cholesterol content, which modulates the level of the mature active form of sterol regulatory element binding proteins (SREBPs), transcription factors for the LDL receptor (Brown and Goldstein 1998). These parameters were therefore investigated as in Chapter 3. For this purpose, human HepG2 liver cells, known to express LDL receptors amenable to regulation (Havekes *et al.*, 1987), were cultured in the presence of increasing amounts of purified EGCG.

4.2 Materials and Methods

4.2.1 Materials

The (-)- Epigallocatechin gallate (EGCG) was purchased from Sigma Chemical Company, Castle Hill, NSW, Australia.

4.2.2 HepG2 Cell Culture

HepG2 cells were grown in monolayer cultures to near confluency in 75 cm^2 flasks with 10 ml of Dulbecco's modified Eagle's media (DMEM) containing 10% (v/v) fetal calf serum (DMEM/FCS) at 37°C with 5% CO_2 as described in Section 2.1. Cells were then incubated for 24 h in 75 cm^2 flasks with 10 ml of DMEM/FCS containing different amounts (0-200 μM) of purified EGCG. Three flasks of cells were treated with each dose of purified EGCG (Section 2.3).

4.2.3 LDL Receptor Binding Activity and LDL Receptor Protein

Following incubation, the cells from each flask were harvested, resuspended in phosphate buffered saline (PBS) and the protein content was determined (Lowry *et al.*, 1951). Determination of the specific LDL receptor binding activity was measured as described in Section 2.4. Cells from this experiment were then frozen at –80°C for at least 24 h. For experimentation, these cells were defrosted slowly, homogenised and the relative amounts of LDL receptor protein were determined by Western blotting with a polyclonal anti-LDL receptor antibody as described in section 2.5.

4.2.4 SREBP-1c

Five 75cm^2 flasks/treatment group were harvested and the cells from these 5 flasks were pooled. Cells were fractionated and the relative amounts of both the inactive precursor and active transcription factor forms of SREBP-1c protein were determined as described in Section 2.7.

4.2.5 Cholesterol, Lathosterol and Chenodeoxycholic Acid.

Cells were frozen at -80°C for at least 24 h and slowly thawed for analysis. Thawed cells were pelleted by centrifugation for 5 min at 400 x *g*. They were then homogenised and protein content determined (Lowry *et al.*, 1951). Total cholesterol (esterified plus unesterified), unesterified cholesterol and lathosterol (index of cholesterol synthesis) were then measured on the homogenised cells as described in Section 2.8. The cholesterol and lathosterol concentrations were expressed relative to the amount of cell protein (mg/mg cell protein and μg/mg cell protein respectively).

The cholesterol and chenodeoxycholic acid concentrations in the media were also determined. For cholesterol, 10 ml of the media was reduced down to near dryness using a

Savant SpeedVac SC100 (Selby Anax, Adelaide, Australia), resuspended in 1 ml of water and analysed as for the cells (Section 2.8). For chenodeoxycholic acid, the cells were grown to near confluency in 75 cm^2 flasks as described above except that the green tea incubations (24 h) were done in 10 ml of DMEM media free of phenol red (Axelson *et al.*, 1991). After the 24 h incubation, the media from 2 flasks was combined (20 ml/sample) and chenodeoxycholic acid was quantified as described in Section 2.9.

4.2.6 Statistical Analysis

Results are expressed as mean ± SEM. Statistical evaluation was done using either linear regression (SPSS software) or a one way ANOVA with Fishers least significant difference (LSD) post hoc test of significance. A value of $p<0.05$ was the criterion of significance.

4.3 Results

4.3.1 EGCG and the LDL Receptor

HepG2 cells were incubated with different concentrations (0, 10, 25, 50, 100 and 200 μM) of EGCG. After treatment a significant positive linear trend was observed between LDL receptor binding activity and the dose of EGCG incubated with the cells ($r^2 = 0.76$, $p < 0.05$) (Figure 4.1A). EGCG treatment was also found to significantly increase the LDL receptor binding activity at the 50 and 200 μM dose compared to the control cells ($p < 0.05$). This increase was up to 3 fold greater in the highest dose treatment group of 200 μM compared to the control. There was no significant trend between the relative amounts of LDL receptor protein and the dose of EGCG incubated with the cells. This was because EGCG treatment significantly increased the LDL receptor protein levels by 2.5 fold over the control in the lower dose group of 25 μl and then attained a plateau through to the highest dose treatment group of 200 μl ($p <$

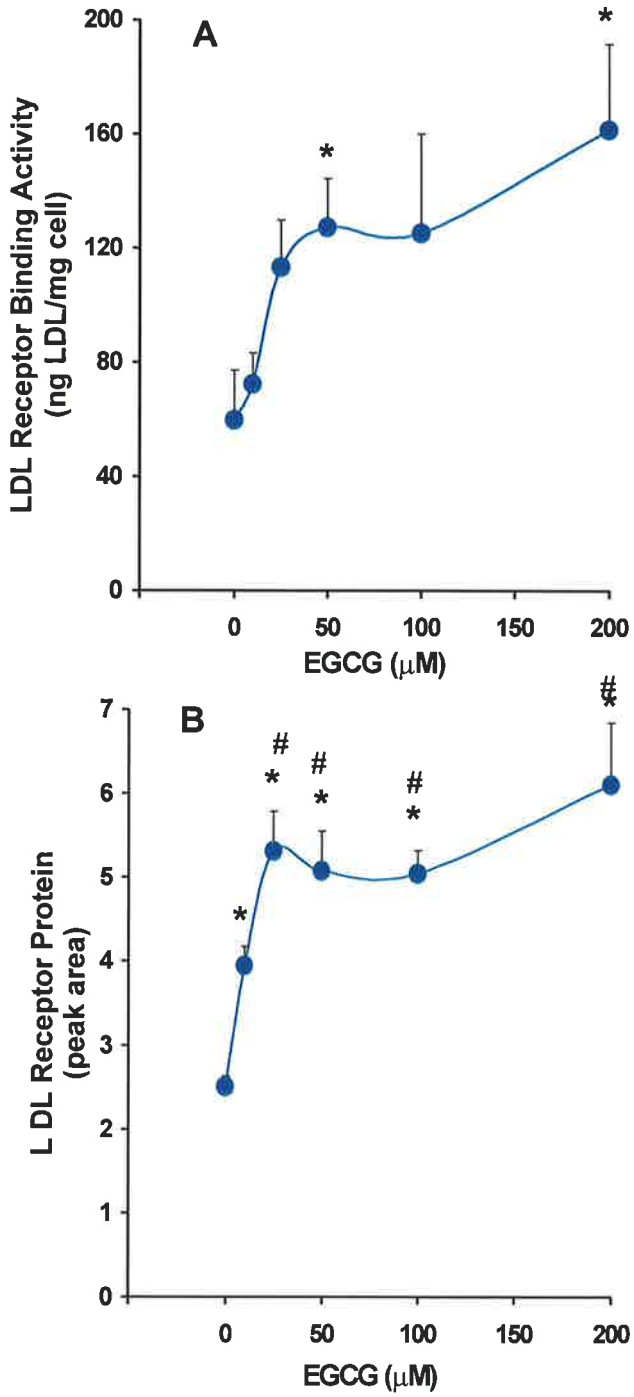


Figure 4.1. Dose dependent effect of EGCG on (A) LDL receptor binding activity and (B) protein. HepG2 cells were incubated for 24 h with increasing amounts of EGCG, 0-200 μM in 10 ml of media. The LDL receptor binding activity was measured as the calcium-dependent binding of LDL-gold to the intact cells. The LDL receptor protein was measured using a polyclonal antibody against the LDL receptor and the ECL western blot method. The values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers LSD ($p < 0.05$). The ($^{\#}$) denotes a significant difference compared to the control using a one way ANOVA and the Bonferroni post hoc test of significance ($p < 0.05$).

0.01) (Figure 4.1B). It was found, however, that there was a significant positive linear trend between LDL receptor protein levels and the log of the dose of EGCG ($r^2 = 0.911, p < 0.01$).

4.3.2 EGCG and Cellular Cholesterol

There was a significant inverse linear trend between total intracellular cholesterol and the dose of EGCG incubated with the cells ($r^2=0.65, p < 0.05$). Treatment with EGCG was also found to significantly decrease intracellular total cholesterol concentrations at 50 and 200 μM compared to control cells ($p < 0.05$) (Figure 4.2). This decrease was up to 28% in the highest dose treatment group of 200 μM compared to the control. There was, however, no linear trend between intracellular unesterified cholesterol concentrations and the dose of EGCG incubated with the cells. There were also no significant reductions noted in intracellular unesterified cholesterol concentrations after EGCG treatment (Figure 4.2).

4.3.3 EGCG and SREBP-1c

Treatment with 200 μM of EGCG resulted in a +42% (150 μg of protein) and +56% (200 μg of protein) increase in the active transcription factor form of SREBP-1c (nuclear cell fraction), compared to the control for the 150 μg and 200 μg of cells respectively. In addition to this, EGCG treatment decreased the inactive precursor form of SREBP-1c (membrane fraction) to undetectable levels (Figure 4.3).

4.3.4 EGCG, Cholesterol Synthesis, Media Cholesterol and Chenodeoxycholic Acid

The cellular lathosterol concentration, measured as an index of cholesterol synthesis, was significantly reduced in the lowest dose treatment group of 50 μM (Figure 4.4A). However, there was no significant difference from control at the higher dose of 100 μM and at 200 μM there was a significant increase (+50%) in the cellular lathosterol concentration (Figure 4.4A).

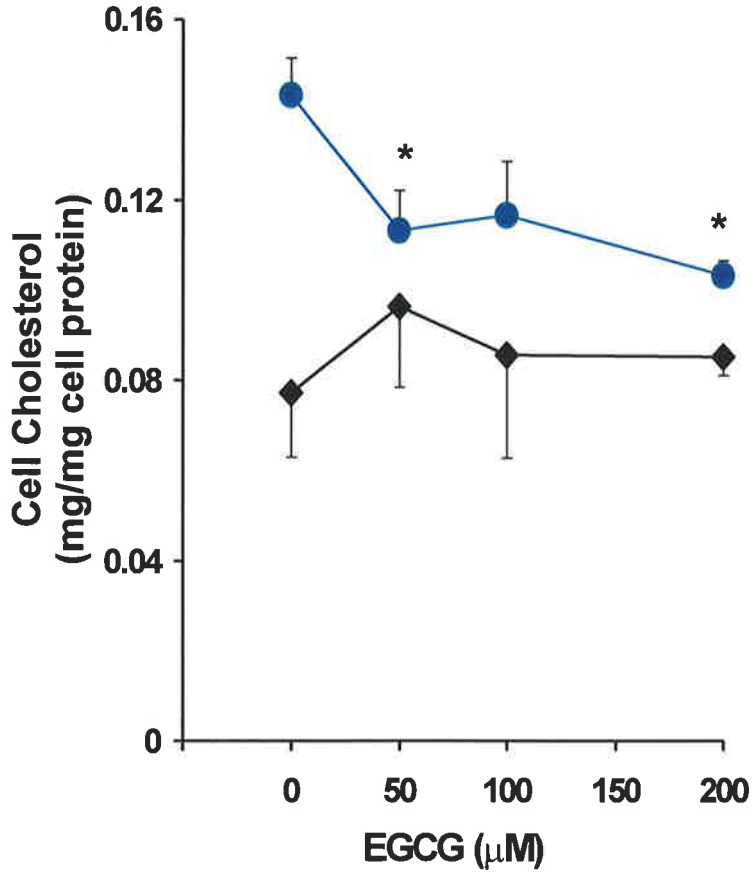


Figure 4.2. Dose-dependent effects of EGCG on intracellular total and unesterified cholesterol concentrations. The HepG2 cells were incubated for 24 h with increasing amounts of EGCG, 0-200 μM in 10 ml of media. Homogenised cells were extracted with hexane and total (●) and unesterified (◆) cholesterol were analysed using gas chromatography and expressed relative to cell protein. The values are means ± SEM of triplicate cell incubations. (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers LSD ($p < 0.05$).

EGCG (μM)	0				200			
Cell Protein (μg)	150	200	150	200	150	200	150	200

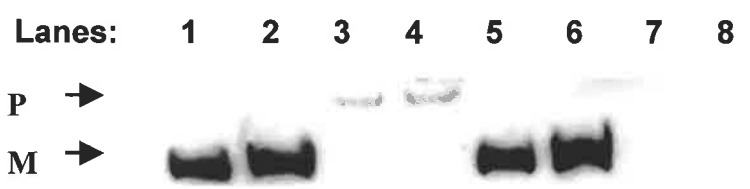


Figure 4.3. The effect of EGCG on SREBP-1c protein. HepG2 cells were incubated for 24h with either 0 (Control, Lanes 1-4) or 200 μM (Lanes 5-8) EGCG in 10 ml of media. Cell extracts (150 and 200 μg) of nuclear (Lanes 1-2,5-6) and microsomal membrane (Lanes 3-4,7-8) fractions were subjected to electrophoresis on an 8% SDS PAGE gel and electrotransfered onto nitrocellulose. The SREBP-1c precursor (**P,** from microsomal membrane fraction) and mature (**M**, from nuclear fraction) forms were detected using a polyclonal antibody and the ECL western blot method. The values are means ± SEM of triplicate cell incubations.

At the lower doses of 50 and 100 μM in 10 ml of media, EGCG did not affect the cholesterol concentration in the media compared to the control (Figure 4.4B). At the highest dose of 200 μM however, EGCG caused a significant increase (+30%) in the media cholesterol concentration (Figure 4.4B). There was a high correlation ($r=0.773$, $p<0.01$) between cell lathosterol concentration (Figure 4.4A) and media cholesterol concentration (Figure 4.4B).

The EGCG treatment did not produce any significant changes in the concentration of chenodeoxycholic acid in the media (Figure 4.4B). The lowered intracellular cholesterol concentration (Figure 4.3) was therefore not likely to be due to an increase in the conversion of cholesterol to bile acids.

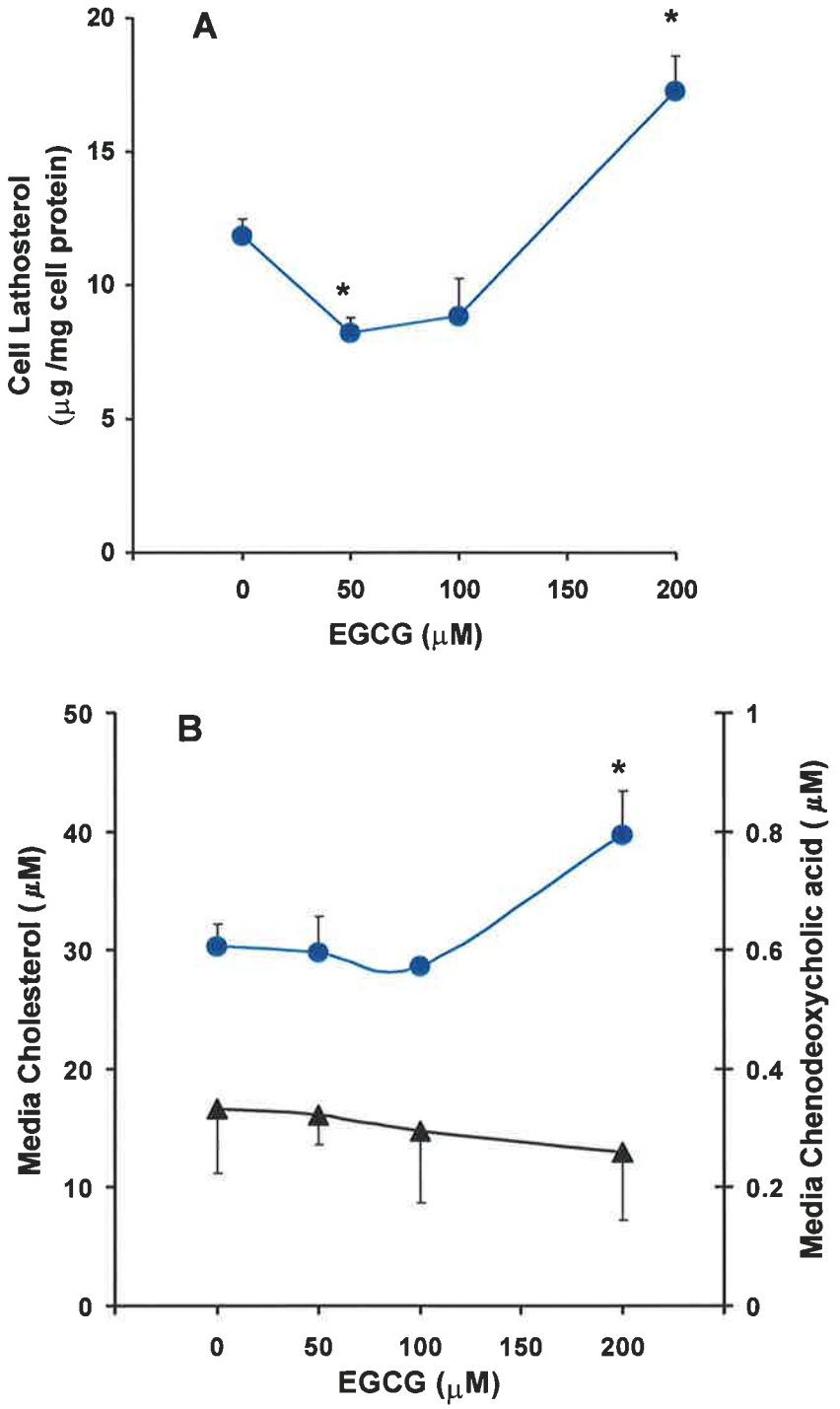


Figure 4.4 (**A**) Dose dependant effect of EGCG on cholesterol synthesis. HepG2 cells were incubated for 24 h with increasing amounts of EGCG, 0-200 µM in 10 ml media. Lathosterol was extracted from homogenised cells and measured using gas chromatography. (**B**) Media cholesterol (●) and chenodeoxycholic acid (▲) were determined using gas chromatography. Values are means ± SEM of triplicate cell incubations. The (*) denotes a significant difference compared to control using a one-way ANOVA and Fishers LSD ($p <$ 0.05).

4.4 Discussion

With the exception of intracellular unesterified cholesterol, treatment with EGCG was able to modulate cholesterol metabolism in the same way as freshly brewed green tea in HepG2 cells. Similarly to green tea, EGCG significantly decreased intracellular total cholesterol concentrations, increased the conversion of SREBP-1c to its mature active form, produced a biphasic "down then up" effect on cholesterol synthesis and increased cholesterol concentrations in the media in the highest dose treatment group. In addition to this, the present study found that EGCG also significantly increased LDL receptor binding activity and protein levels above the control, which is in agreement with previous studies (Bursill 1996, Pal *et al.*, 1999) and with the effect to green tea. This increase in the LDL receptor by EGCG may explain its hypocholesterolaemic effects as found in animal intervention studies (Matsuda *et al.*, 1986, Fukuyo *et al.*, 1986, Chisaka *et al.*, 1988). It may also explain the hypocholesterolaemic effects of green tea extracts in epidemiological and animal intervention studies because EGCG is the most abundant ingredient in green tea. Taken together, these findings provide evidence that EGCG is the active component in green tea that modulates cholesterol metabolism.

As with green tea, when cells were incubated with EGCG, cellular total cholesterol concentrations were 30% lower than in the control cells and there was an increase in the conversion of SREBP-1c to its mature active transcription factor form. In contrast to green tea, however, EGCG treatment did not lower cellular unesterified cholesterol and therefore the reductions in cellular cholesterol were likely to be in the esterified form of cholesterol. This indicates that whilst EGCG appears to increase the LDL receptor via the same mechanism as green tea, i.e. by increasing in the activation of SREBP-1c, it appears that the whole cell unesterified cholesterol as measured in the study is not the regulatory pool. Esterified cholesterol is thought to be the inactive form of cholesterol and not involved in regulatory processes and it is

the unesterified form of cholesterol which is thought to be regulatory (Winegar *et al.*, 1996). Unesterified cholesterol can be converted to oxysterols (Grundy 1991), which regulate the activation of SREBP's by inhibiting their proteolytic cleavage from the endoplasmic reticulum (Brown and Goldstein 1998). Normally, if cleaved, it releases the active transcription factor form of SREBP's which can bind upstream of the LDL receptor and activate transcription (Brown and Goldstein, 1997). As mentioned, treatment with EGCG did not, however, change cellular unesterified cholesterol concentrations. This indicates that either the regulatory pool of unesterified cholesterol is not measurable in these cells (Havekes *et al.*, 1987) or that EGCG may have directly increased the proteolytic cleavage of SREBP-1 to its active transcription factor form rather than via a reduction in oxysterols.

It appeared that treatment with EGCG decreased total cellular cholesterol concentrations via the same mechanisms as freshly brewed green tea (Chapter 3). Similarly to green tea, at the lowest concentration of EGCG (50 μM) there appeared to be a decrease in cholesterol synthesis as measured using cell lathosterol, an index of cholesterol synthesis (Kempen *et al.*, 1988). At this concentration, EGCG may have inhibited cholesterol synthesis which, in turn, may have contributed to the reduction in cell cholesterol. One possible mechanism by which EGCG may have lowered cholesterol synthesis is to inhibit HMGCoA reductase, the rate-limiting enzyme in cholesterol synthesis (See Sections 5.4 and 6.1.1).

In contrast to the lower dose concentration (50 μM), cholesterol synthesis appeared to be significantly higher in the group treated with 200 μM EGCG. Despite this, however, cholesterol did not accumulate within the cells, as the cell cholesterol decreased by 28%. Rather than having accumulated in the cells, the extra cholesterol seemed to be in the media, where its concentration was increased by +30% over the control. It therefore appears that at the highest concentration,

EGCG increased the efflux of cholesterol from the cells into the media to such an extent that it caused an increase in cholesterol synthesis but this did not fully compensate for the loss of cell sterol. There was a high correlation ($r = 0.773$) between cell lathosterol and the concentration of cholesterol in the media, suggesting that the concentration of cholesterol in the media was directly linked to the amount of cholesterol synthesised by the cells. The increase seen in the SREBP-1c mature form with 200 μM EGCG is also consistent with the observed increase in cholesterol synthesis as SREBP-1 also upregulates the HMG-CoA reductase gene, the rate limiting enzyme in cholesterol synthesis.

Treatment with EGCG did not appear to increase the conversion of cell cholesterol into bile acids (Axelson *et al.*, 1991), as the chenodeoxycholic acid content of the media did not change significantly. This therefore would not have contributed to the decrease in cellular cholesterol.

As hypothesised for the green tea treatment (Section 3.4), the ability of EGCG to form complexes with cholesterol (Ikeda *et al.* 1992) may explain the effects seen *in vitro* with the highest dose of EGCG (200 μM) in the present study. At this concentration EGCG may complex with enough cholesterol in the media to render it essentially cholesterol-deficient. Cholesterol could then move from the cells into the media by normal diffusion down the concentration gradient. This could explain why the concentration of cholesterol is seen to increase in the media while the cells are not able to regain their normal intracellular cholesterol levels despite an increase in cholesterol synthesis.

The treatment with EGCG produced the same effects on cholesterol synthesis, cholesterol concentrations in the media and bile acids as freshly brewed green tea. This indicates that EGCG

lowers intracellular total cholesterol concentrations in the same fashion as green tea and indicates that it is the active ingredient in green tea that modulates this aspect of cholesterol metabolism.

In conclusion, with the exception of its effects on cellular unesterified cholesterol, the effects of EGCG on cholesterol metabolism in HepG2 cells were found to be so similar to green tea that it is likely to be the active ingredient. EGCG treatment increased the activation of SREBP-1c and therefore appeared to upregulate the LDL receptor via the same mechanism as green tea. The way in which EGCG treatment lowered cellular total cholesterol concentrations was also similar to green tea. This appeared to be by inhibiting cholesterol synthesis at the lower dose (50μM) and increasing the efflux of cholesterol from the cells into the media at the highest dose (200μM). Overall, the results from Chapter 3 and the present study have demonstrated that green tea and EGCG can modulate cholesterol metabolism *in vitro* in HepG2 cells. Whether these effects are relevant *in vivo* was investigated in the following chapter (Chapter 5). For this purpose, hypercholesterolaemic rabbits were fed a green tea extract, enriched in catechins, to determine if the effects of green tea and EGCG on cholesterol metabolism *in vitro* can be translated into a more physiological model.

Chapter 5

A GREEN TEA EXTRACT LOWERS PLASMA CHOLESTEROL IN THE HYPERCHOLESTEROLAEMIC RABBIT.

5.1 Introduction

Inhibition of cholesterol absorption has been proposed in the literature as a mechanism to explain the cholesterol-lowering effects of green tea. This is because the faecal excretion of total lipids and cholesterol were found to be higher in animals consuming green tea extracts (Muramatsu *et al.*, 1986, Fukuyo *et al.*, 1986, Matsuda *et al.*, 1986, Chan *et al.*, 1999). The EGCG has also been observed to inhibit the uptake of ^{14}C-cholesterol from the intestine (Chisaka *et al.*, 1988). This apparent reduction in intestinal cholesterol absorption has been ascribed to EGCG reducing the solubility of cholesterol into mixed bile salt micelles (Ikeda *et al.*, 1992). It has also recently been found that hamsters and rats fed green tea extracts had increased faecal excretion of bile acids (Chan *et al.*, 1999, Yang and Koo 2000)

This apparent inhibition of cholesterol absorption and bile acid reabsorption by green tea should lead to a reduction in liver cholesterol concentrations. In order to compensate for this it would be expected that LDL receptor activity and cholesterol synthesis in the liver would increase (Brown and Goldstein 1986). These effects have been noted in studies using inhibitors of cholesterol absorption such as tiqueside (Harwood *et al.*, 1993) and inhibitors of intestinal reabsorption of bile acids such as cholestyramine (Dory *et al.*, 1990, Rudling 1992). This was also mimicked in the *in vitro* studies where both green tea and EGCG increased the LDL receptor and increased cholesterol synthesis in the highest dose treatment group (Chapters 3 and 4). The increase in the LDL receptor can mediate the lowering of plasma cholesterol by enhancing the uptake of LDL cholesterol from the circulation. However, the cholesterol-lowering potential of these agents can be offset by an increase in cholesterol synthesis (Brown and Goldstein 1986).

Indirect evidence that the LDL receptor may be upregulated *in vivo* by green tea extracts was found in a study by Chisaka *et al.* (1988). This study found that the administration of EGCG to rats enhanced the removal of intravenously injected ^{14}C-cholesterol from the plasma. An increase in the plasma clearance of cholesterol may be due to upregulation of the LDL receptor. The hypocholesterolaemic effects of green tea and green tea extracts may also be due to a more direct effect on the LDL receptor. Support for this has been found *in vitro* where freshly brewed green tea (Chapter 3), a crude catechin extract (Pal *et al.*, 1999) and purified EGCG (Chapter 4) increased the hepatic LDL receptor of HepG2 cells. Furthermore, studies in our laboratory found that the administration of a crude catechin extract to rats for 12 days increased LDL receptor binding activity and protein, providing direct evidence that this effect is physiologically relevant (Bursill 1996).

Little is known about the effects of green tea on cholesterol synthesis. Animal intervention studies by Chan *et al.* (1999) and Yang and Koo (2000) have found no effect of green tea on the "*in vitro*" activity of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in hamsters and rats. The HMG-CoA reductase enzyme catalyses the rate-limiting step in cholesterol biosynthesis, but measurement of its activity "*in vitro*", however, may not always reflect the level of cholesterol synthesis (Section 1.12.3). *In vitro* studies in HepG2 cells have found, however, that freshly brewed green tea (Chapter 3) and EGCG (Chapter 4) significantly lowered lathosterol concentration, an index of cholesterol synthesis, in the lower dose treatments and increased it in the higher dose concentrations.

In summary, evidence from the literature suggests that green tea and green tea extracts may lower plasma cholesterol *in vivo* by inhibiting cholesterol absorption which is likely to result in increase in the LDL receptor and an increase in cholesterol synthesis. Green tea (Chapter 3) and EGCG (Chapter 4) have also been found to increase LDL receptor binding activity and protein levels, as well as producing a biphasic "down then up" effect on cholesterol synthesis. If green tea and EGCG have the same effects *in vivo* it may explain the hypocholesterolaemic effects found with green tea extracts and purified catechins in epidemiological and animal intervention studies (Section 3.1). This is because an increase in the LDL receptor and a decrease in cholesterol synthesis (found in the lower dose treatments) can contribute to lowering plasma cholesterol concentrations. The aims of this study were therefore to determine if the effects of freshly brewed green tea and EGCG on cholesterol metabolism *in vitro* can be translated *in vivo* in the hypercholesterolaemic rabbit model, thereby providing a mechanism for their plasma cholesterol-lowering properties.

5.2. Materials and Methods

5.2.1 Catechin Extract

The crude catechin extract was prepared from commercially available "Special Gunpowder" green tea, packaged by the China National Native Products and Animal By-products Import and Export Corporation, Zhejiang Tea Branch, China. The method used was based on the method of Huang *et al.* (1992) (Figure 5.1). Briefly, 15 kg of green tea was extracted with 3 volumes (v/w) of methanol at 50°C for 3 h. Solvent was removed from the extract using a reduced pressure rotary evaporator. The residue was dissolved in

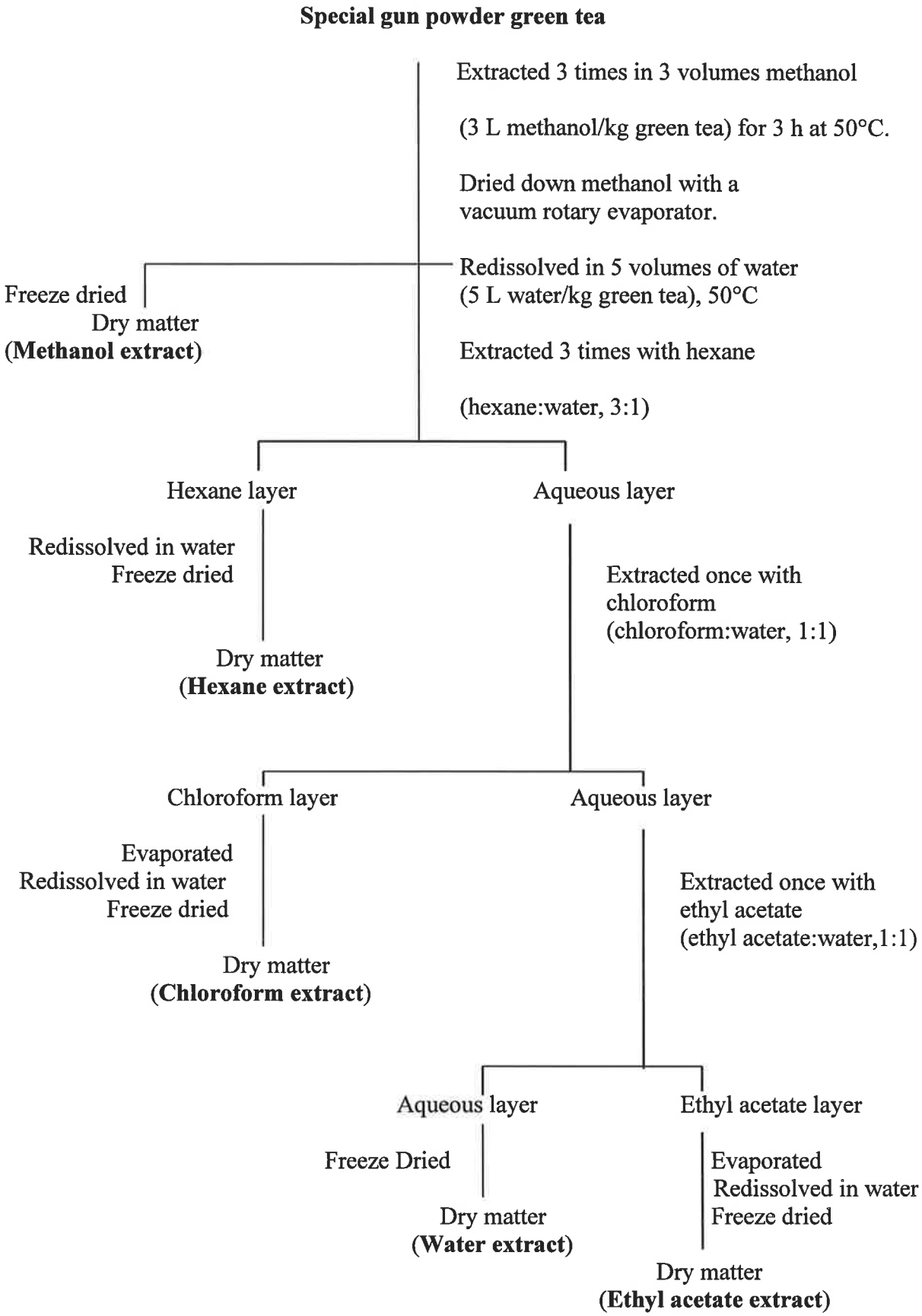


Figure 5.1. Green tea extraction

2 volumes of water (v/w) at 50°C and extracted twice with equal volumes of hexane (v/v) and once with an equal volume of chloroform (v/v). The remaining aqueous phase was then extracted once with an equal volume of ethyl acetate (v/v) which extracts the polyphenolic compounds including the catechins. The ethyl acetate was then evaporated, the residue redissolved in the minimum amount of warm water (50°C) and freeze dried.

5.2.2. Animal Study

Twenty-four (4 month old) male New Zealand White rabbits (IMVS, Gillies Plains, SA, Australia) were housed in individual cages at the CSIRO Health Sciences and Nutrition animal facility (Kintore Avenue, Adelaide, SA, Australia). Ethics approval for the study was obtained from the University of Adelaide and CSIRO Health Sciences and Nutrition Animal Ethics Committees. The rabbits were housed individually in surroundings of controlled temperature (20 ± 1°C) and a 12 h light cycle (06:00 to 18:00).

All rabbits were initially fed a diet containing 0.25% (w/w) cholesterol that was mixed with their basic rabbit chow (IMVS, Gillies Plains, SA). This diet was fed to the rabbits for a period of 2 weeks to increase their plasma cholesterol concentrations prior to the administration of the crude catechin extract.

The rabbits were then allocated into 4 different treatment groups, based on their plasma cholesterol levels and body weight. The crude catechin extract was fed at concentrations of 0, 0.5, 1 or 2 % (w/w). The extract was mixed in their normal rabbit chow along with 0.25% (w/w) cholesterol and fed to the rabbits for a period of 28 days. Daily consumption of the diets was determined.

Rabbits were fasted overnight and blood samples for lipid analysis were taken from the ear artery prior to and after the two-week cholesterol-only feeding period. Following the 4 weeks of dietary intervention with the crude catechin extract, the rabbits were fasted overnight and the following morning were injected intramuscularly (IM) with 1.2 ml (2.5 mg) of Acepromazine. Once sedated (approximately 30 min), the rabbits were injected IM with a muscle relaxant (0.75 ml Rompun, 15 mg) and a general anaesthetic (1.5 ml Ketamine, 150 mg). Under deep anaesthesia the rabbits were bled by cardiac puncture until euthanasia. Blood was collected into EDTA tubes (1 mM) and plasma was isolated by centrifugation at 3000 x *g* for 10 min at 4°C. The entire aorta, from the ascending arch to the ileac bifurcation, was carefully removed and divided into 3 segments: the aortic arch, the descending aorta and the abdominal aorta. The aortic arch and the abdominal aorta were fixed and stained for atheroma assessment then quantified using TM/TC Image Analysis Systems (Digithurst, Herts, England) and MicroScale software. The remaining descending thoracic aorta was frozen in liquid nitrogen and stored at -80° for determination of artery cholesterol. Livers were also excised, then weighed, frozen in liquid nitrogen and stored at -80°C.

5.2.3. Plasma Lipids

Plasma cholesterol and triglyceride concentrations were measured on a Cobas Bio automated centrifugal analyser (F. Hoffmann-LaRoche, Basel, Switzerland) using enzymatic test kits (Roche Diagnostica, Basel, Switzerland).

Lipoprotein fractions containing VLDL + IDL ($1.006 < d < 1.019$), LDL ($1.019 < d < 1.063$ g/ml) and HDL ($1.063 < d < 1.21$ g/ml) were isolated by sequential ultracentrifugation (Havel *et al.*, 1955), using an Optimal TLX benchtop ultracentrifuge

and Beckman TLA 120.2 rotor (Beckman Instruments, CA, USA), from 1 ml of fasting rabbit plasma obtained after treatment with the green tea extract. The cholesterol and triglyceride and protein content of the different lipoprotein fractions were then determined using the Cobas Bio. The protein content of the lipoprotein fractions was also determined on the Cobas Bio using the method of Clifton *et al.* (1988).

5.2.4. Cholesterol Synthesis and Intrinsic Capacity to Absorb Dietary Cholesterol

Plasma lathosterol and phytosterols (campesterol and β-sitosterol) were measured by gas chromatography (GC) as described by Wolthers *et al.* (1991). The ratios of serum lathosterol and phytosterol concentrations in the plasma to the plasma cholesterol concentration, have been found to correlate with whole body cholesterol synthesis (Kempen *et al.*, 1988) and the intrinsic capacity to absorb dietary cholesterol (Tilvis *et al.*, 1986) respectively.

5.2.5. Hepatic LDL Receptor Binding Assay

5.2.5.1 *Preparation of Soluble Rat Liver Membrane Proteins*

Solubilised liver membranes were prepared from rabbits livers as described previous by Kovanen *et al.*, (1979) with one exception. Five times the concentration of phenylmethylsulfonyl fluoride (5 mM) and N-ethylmaleimide (5 mM) were added to both the homogenisation and solubilisation buffer. This was done to prevent the degradation and dimerisation of the LDL receptor protein as the rabbit hepatic LDL receptor seemed more susceptible to this compared to the rat.

Homogenisation: A 2-3 g piece of liver was homogenised in 10 ml of homogenisation buffer (10 mM Tris-HCl, 0.154 M NaCl, 2 mM $CaCl_2$, 5 mM phenylmethylsulfonyl fluoride [PMSF] and 5 mM N-ethylmaleimide, pH 7.5) by pulsing for 2 x 10 sec with a

Ultra-Turrax homogeniser (Janke and Kunkel, John Morris Scientific, Sydney, Australia). Liver that was not homogenised was spun down by centrifugation for 5 min at 500 x *g*. The supernatant was collected and spun at 8000 x *g* for 15 min in a JA-21 rotor to pellet the mitochondrial liver fraction. The resultant supernatant was then spun at 100K x *g* for 1 h to pellet the liver plasma and microsomal membranes.

Solubilisation: The membrane pellet was resuspended in 1.5 ml of solubilisation buffer (250 mM Tris-maleate, 2 mM $CaCl_2$, 5 mM PMSF, 5 mM N-ethylmaleimide, pH 6.0) using a pasteur pipette. This was sonicated for 2 x 20 sec pulses before adding an equal volume (1.5 ml) of 2% (v/v) Triton-X 100 with 2 mM $CaCl_2$ (Kovanen *et al.*, 1979), then agitated for 30 mins on a rotating wheel at 4°C. Triton-X 100 was removed from the solution by adding thoroughly washed Amberlite XAD-2 (0.5 g/ml) and agitating for a further 1 h on a rotating wheel (Roach *et al.*, 1985). Following this, the Amerberlite was allowed to settle and the supernatant was collected and clarified by centrifugation at 10K x *g* for 10 mins. The protein content of the solubilised liver membrane solution was determined (Lowry *et al.*, 1951).

5.2.5.2 Determination of LDL Receptor Binding Activity

To measure LDL receptor binding activity, 8 µg of the solubilised liver membranes were applied to nitrocellulose paper using a slot blot apparatus (Schleicher and Schuell, Westborough, MA). The nitrocellulose was then blocked with a 1:1 dilution of blocking buffer (8% w/v) bovine serum albumin (BSA), 240 mM Tris-HCL, 100 mM NaCl and 8 mM $CaCl_2$, pH 8.0) as described by Roach *et al.* (1993). The nitrocellulose membranes were then incubated in incubation buffer (1:3, blocking buffer:water) containing either 20 µg/ml LDL-gold in the absence and presence of 20 mM EDTA to determine total and non-specific binding, respectively. The nitrocellulose paper was soaked in water for 30

min and then incubated with IntenSE BL silver enhancement kit (Amersham, UK) for a further 30 min. This was washed with water, dried and scanned using an LKB Ultrascan XL enhanced laser densitometer (Pharmacia LKB Biotechnology, North Ryde, NSW, Australia). The specific binding (total minus the non-specific binding) was taken to be the LDL receptor binding activity which is expressed as peak height, determined from the laser densitometer scan.

5.2.6 Quantification of LDL Receptor Protein

Relative amounts of LDL receptor protein were also determined. Solubilised rabbit liver membranes (100 μg) were prepared as described in Section 5.2.5.1 and were subjected to electrophoresis on 3-15% SDS polyacrylamide gradient gels (Laemmli et al., 1970) and electrotransferred onto nitrocellulose paper (Burnette *et al.*, 1981). The membranes were then overlaid with a polyclonal antibody against the LDL receptor followed by an anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO USA) as described in Section 2.5.3. The LDL receptor band was then detected on X-ray film (Hyperfilm-ECL, Amersham, North Ryde, NSW, Australia) using an enhanced chemiluminescence kit from Amersham. Quantification of LDL receptor protein was performed by laser densitometry. Results are expressed as peak area, determined from the densitometer scan.

5.2.7. Liver Lipid Determinations

Total cholesterol, unesterified cholesterol and triglycerides were measured on the liver homogenate and the solubilised liver membranes. Both liver preparations were initially sonicated, then diluted 1:1 with a 2% (w/w) Triton X-100 and 2 mM $CaCl_2$ solution. This was agitated on a rotating wheel for 30 mins at 4°C and protein content determined

(Lowry *et al.*, 1951). Lipid measurements were performed using enzymatic methods on the Cobas Bio and expressed relative to the protein concentrations.

2.8. Artery Cholesterol Measurements

The total cholesterol in the descending aorta was determined on approximately 15 to 20 mm segments of aorta, weighing 0.3-0.5 g, which were homogenised in 2 ml of buffer (10 mM Tris-HCL, 154 mM Na Cl, 2 mM $CaCl_2$ and 1 mM PMSF, pH 7.5) and then sonicated on ice for 30 sec. Cholesterol was extracted by the Folch method (Folch *et al.*, 1957) and then subjected to saponification. Briefly, standards were prepared for cholesterol (20-200 μg/ml) and 5β-cholestan-3α-ol (0.5 mg/ml, internal standard). Thirty μl of internal standard was added to each tube and the cholesterol standards were added to the standard tubes then dried under a stream of nitrogen. One ml of aorta homogenate was added to the sample tubes and mixed with 4 ml of 2:1 (v/v) chloroform : methanol. The chloroform layer was collected and evaporated to dryness under a stream of nitrogen. Cholesterol was hydrolysed by incubating with 100 μl of 33% (v/v) aqueous KOH and 2 ml ethanol in a water bath set a 60°C for 30 min. The cholesterol was then extracted from the aqueous phase using 2 ml of hexane, dried under a stream of nitrogen and redissolved in 50 μl of hexane for GC injection.

The GC conditions were the same as described in Section 2.8.4.

5.2.9. Statistical Analysis

All values are expressed as the mean ± standard error of the mean (SEM). Data was analysed using a linear regression or a one way analysis of variance (ANOVA) with the

Fishers least significant difference (LSD), Scheffe or Bonferroni tests of significance where appropriate. A value of $p<0.05$ was the criterion of significance.

5.3 Results

5.3.1. Daily Food Consumption

Overall the daily dietary intake was: 116 ± 10.36 g of diet per day per rabbit. Rabbits in the treatment groups consumed: 115.6 ± 12.13, 105.4 ± 7.29, 117.3 ± 17.38 or 125.7 ± 15.98 g/day for the 0, 0.5, 1 and 2% (w/w) groups respectively and these amounts were not significantly different.

5.3.2. Plasma Lipids

Plasma cholesterol levels at the beginning of the study were not significantly different between treatment groups: 1.03 ± 0.16, 0.94 ± 0.12, 0.89 ± 0.13 and 0.88 ± 0.12 mmol/L (mean ± SEM) cholesterol concentration for the 0, 0.5, 1 and 2% (w/w) treatment groups respectively. Neither were they significantly different between groups after the 2 weeks of cholesterol-only feeding: 4.82 ± 0.84, 5.38 ± 1.1, 3.67 ± 0.80 and 3.44 ± 0.107 mmol/L. The average cholesterol concentration was, however, significantly increased from 0.91 ± 0.06 to 4.26 ± 0.43 mmol/L (p for ANOVA <0.001) after two weeks of feeding 0.25% (w/w) cholesterol.

After administration of the crude catechin extract along with 0.25% (w/w) cholesterol for 4 weeks there was a significant inverse linear trend between plasma cholesterol and the dose of crude catechin extract administered ($r = -0.50$, $p < 0.05$) (Figure 5.2). Using an ANOVA it was found that the crude catechin extract significantly reduced plasma

cholesterol concentration in the 2% w/w treatment group (-60%, $p < 0.05$) compared to the control. There was no significant linear trend noted between plasma triglyceride levels and dosage where the concentrations for the different treatment groups were: 0.50 ± 0.04, 0.62 ± 0.14, 0.95 ± 0.13 and 0.95 ± 0.23 mmol/L for the 0, 0.5, 1 and 2% (w/w) treatment groups respectively.

5.3.3 Plasma lipoprotein cholesterol

The distribution of cholesterol within the 3 main lipoprotein fractions (VLDL + IDL, LDL and HDL) is shown in Figure 5.3. Administration of the crude catechin extract caused a significant inverse linear trend between LDL cholesterol and the dose of the crude catechin extract ($r = -0.50$, $p < 0.05$). It was found that the crude catechin extract significantly reduced cholesterol in the LDL fraction in the 2% (w/w) treatment group compared to the control (-80%, $p < 0.05$). Administration of the crude catechin extract also produced significant inverse linear trend between cholesterol in the VLDL + IDL fraction and the dose of the crude catechin extract ($r = -0.49$, $p < 0.05$). There was a significant reduction in cholesterol concentration in the VLDL + IDL fraction in the 2% w/w treatment group compared to the control (-65%, $p < 0.05$). No significant linear trend was found between cholesterol in the HDL fraction and the dose of the crude catechin extract (Figure 5.3). The recovery of cholesterol in the lipoprotein fractions isolated from rabbit plasma after administration of the crude catechin extract was approximately 70%.

The protein and triglyceride content in the VLDL + IDL (Table 5.1), LDL (Table 5.2) and HDL (Table 5.3) lipoprotein fractions were not changed after consumption of the crude catechin extract compared to the control.

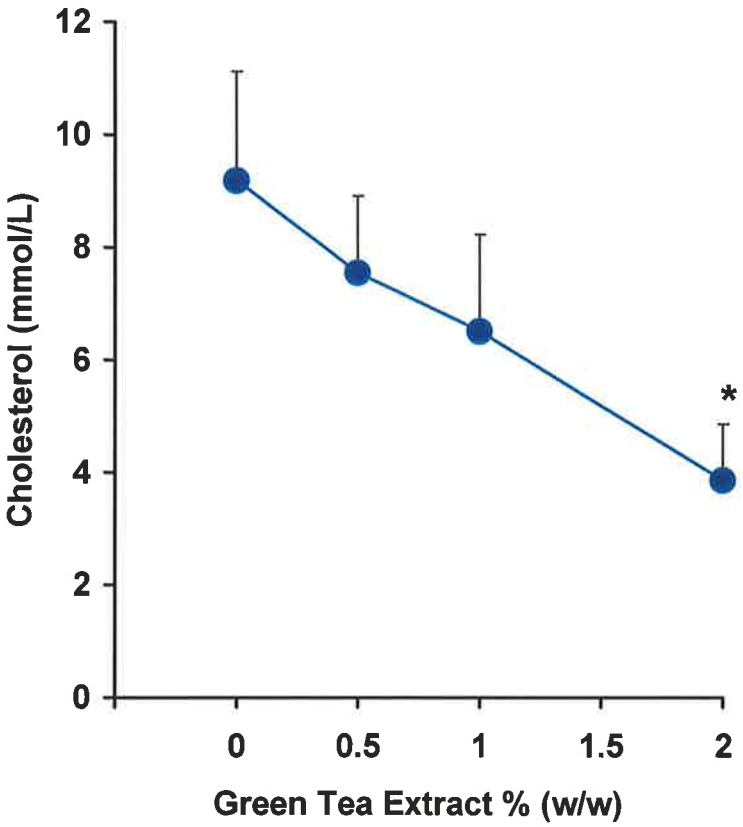


Figure 5.2. Effect of the crude catechin extract from green tea on plasma cholesterol concentrations. Twenty four hypercholesterolaemic rabbits, randomised into 4 treatment groups of 6 rabbits each, were fed a crude catechin extract at concentrations of 0, 0.5, 1 or 2% (w/w) mixed in with normal rabbit chow and 0.25% (w/w) cholesterol for 28 days. Values are expressed as mean ± SEM. (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers LSD ($p < 0.05$).

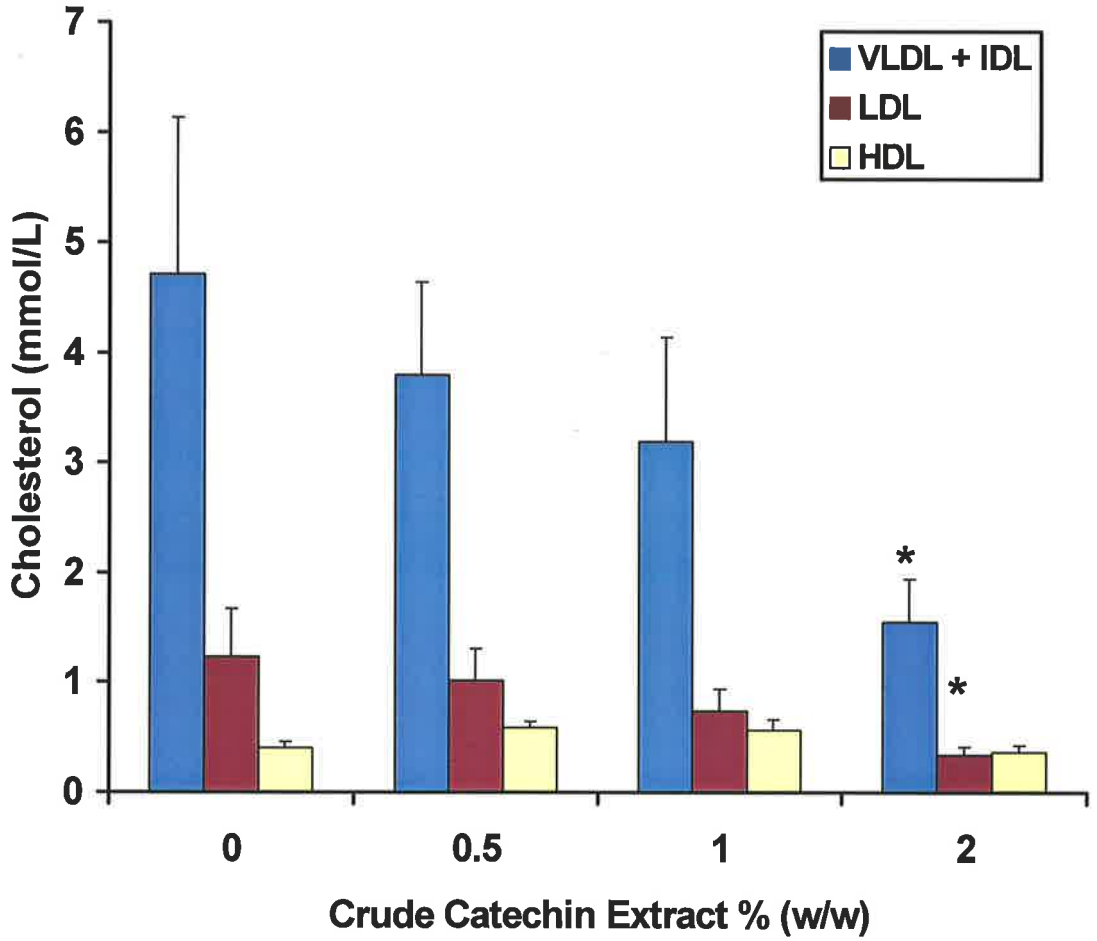


Figure 5.3. Effect of the crude catechin extract from green tea on cholesterol concentrations in lipoprotein fractions. Twenty four hypercholesterolaemic rabbits, randomised into 4 treatment groups of 6 rabbits each, were fed a crude catechin extract at concentrations of 0, 0.5, 1 or 2% (w/w) mixed in with normal rabbit chow and 0.25% (w/w) cholesterol for 28 days. Lipoproteins, VLDL + IDL ($1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$ g/ml) and HDL (d $1.063 < d < 1.21$ g/ml) were isolated from plasma using sequential ultracentrifugation. Cholesterol in the lipoprotein fractions was measured using enzymatic techniques. Values are expressed as mean ± SEM. (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

Table 5.1
Lipid and lipoprotein concentrations in the VLDL + IDL fraction isolated from rabbit plasma following dietary intervention with a crude catechin extract.

	Crude Catechin Extract % (w/w)			
	0	**0.5**	**1**	**2**
Cholesterol (mmol/L)	4.7^{a} ±1.4	3.8^{ab} ±0.84	3.2^{ab} ±0.94	1.6^{b} ±0.39
Triglycerides (mmol/L)	0.22 ±0.02	0.25 ±0.06	0.34 ±0.06	0.21 ±0.04
Protein (g/L)	0.61 ±0.16	0.59 ±0.09	0.51 ±0.11	0.36 ±0.08

Values are expressed as mean ± SEM.

For cholesterol values without common superscripts are significantly different, determined using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

There were no significant changes in triglycerides or protein.

Table 5.2
Lipid and lipoprotein concentrations in the LDL fraction isolated from rabbit plasma following dietary intervention with a crude catechin extract

	Crude Catechin Extract % (w/w)			
	0	**0.5**	**1**	**2**
Cholesterol (mmol/L)	1.2^{a} ±0.44	1^{ab} ±0.29	0.74^{ab} ±0.2	0.34^{b} ±0.07
Triglycerides (mmol/L)	0.08 ±0.02	0.3 ±0.02	0.19 ±0.06	0.16 ±0.04
Protein (g/L)	0.38 ±0.09	0.52 ±0.16	0.33 ±0.05	0.28 ±0.05

Values are expressed as mean ± SEM

For cholesterol values without common superscripts are significantly different, determined using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

There were no significant changes in triglycerides or protein.

Table 5.3
Lipid and lipoprotein concentrations in the HDL fraction isolated from rabbit plasma following dietary intervention with a crude catechin extract

	Crude Catechin Extract % (w/w)			
	0	**0.5**	**1**	**2**
Cholesterol (mmol/L)	0.41 ±0.06	0.59 ±0.05	0.56 ±0.1	0.37 ±0.07
Triglycerides (mmol/L)	0.54 ±0.06	0.74 ±0.14	1.32 ±0.29	0.98 ±0.18
Protein (g/L)	1.1 ±0.1	1.14 ±0.06	1.24 ±0.04	1.81 ±0.42

Values are expressed as mean ± SEM

There were no significant changes in cholesterol, triglycerides or protein.

Table 5.4. Ratios of cholesterol concentrations in lipoproteins isolated from rabbit plasma following dietary intervention with the crude catechin extract for 28 days.

	Crude Catechin Extract % (w/w)			
Ratio of cholesterol in lipoprotein fractions	**0**	**0.5**	**1**	**2**
VLDL + IDL: HDL-cholesterol	11.5^{a} ±2.8	6.5^{ab} ±1.4	5.2^{b} ±1.1	$4.2^{b\#}$ ±0.8
LDL: HDL-cholesterol	3.1^{a} ±1	1.7^{ab} ±0.4	1.2^{b} ±0.2	$1^{b\Psi}$ ±0.2
VLDL + IDL + LDL: HDL-cholesterol	14.6^{a} ±3.7	8.2^{b} ±1.8	6.5^{b} ±1.3	$5.2^{b\#}$ ±0.9

Values are expressed as mean ± SEM.

Values without common superscripts are significantly different, determined using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

($^{\#}$) significantly different compared to the control using a one way ANOVA and Scheffe post hoc test ($p < 0.05$).

($^{\Psi}$) significantly different compared to the control using a one way ANOVA and the Bonferroni post hoc test ($p < 0.05$).

The ratios of VLDL + IDL:HDL-cholesterol, LDL:HDL-cholesterol and VLDL + IDL + LDL:HDL-cholesterol are shown in Table 5.4. There was a significance inverse linear trend between VLDL + IDL:HDL, LDL:HDL and VLDL + IDL + LDL:HDL and the dose of the crude catechin extract (r = -0.57, -0.53 and –0.57 respectively, $p < 0.01$). Significant reductions were observed for the VLDL + IDL:HDL, LDL:HDL and VLDL + IDL + LDL:HDL ratios in the 2% (w/w) treatment group compared to the control group ($p < 0.01$). This indicated an improved atherogenic index with consumption of the crude catechin extract.

5.3.4. Cholesterol in the Arteries

The concentration of cholesterol in the descending thoracic segment of the aorta from rabbits is shown in Table 5.5. There was a significant inverse linear trend between cholesterol in the thoracic aorta and the dose of the crude catechin extract (r = -0.55, $p < 0.01$). There was a significant reduction in total cholesterol in the thoracic aorta of the rabbits fed 2% (w/w) crude catechin extract compared to the control group (-30%, $p < 0.05$). There was, however, no trend between the percent surface area of the aortic arch stained with lipophilic oil red O, and the dose of crude catechin extract administered (Table 5.5).

5.3.5. Liver Lipids

The crude catechin extract resulted in significant inverse linear trends between both total and unesterified cholesterol concentrations in the homogenate and the dose of the crude catechin extract (r = -0.54 and -0.43 respectively, $p < 0.05$). There were significant reductions in total and unesterified cholesterol in the liver homogenate (-25% and –15% respectively) in the 2% (w/w) treatment group compared to the controls

Table 5.5. Cholesterol content and fatty streak assessment in aorta dissected from rabbits following dietary intervention with a crude catechin extract for 28 days.

	Crude Catechin Extract % (w/w)			
	0	**0.5**	**1**	**2**
Thoracic aortic cholesterol (μmol cholesterol/g)	0.98^{a} ±0.13	1.22^{a} ±0.12	0.87^{ab} ±0.05	0.73^{b} ±0.06
Aortic arch fatty streak (% total surface area with Lipophilic stain)	2.35 ±0.73	2.28 ±1.05	1.57 ±0.53	1.93 ±0.67

Values are expressed as mean ± SEM.

Values without common superscripts are significantly different, determined using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

There were no significant changes in aortic arch fatty streak formation.

Table 5.6. Total and unesterified cholesterol and triglyceride concentrations in rabbit liver homogenate and membranes after dietary intervention with a crude catechin extract for 28 days.

	Crude Catechin Extract % (w/w)			
Liver Fraction	**0**	**0.5**	**1**	**2**
Homogenate				
Total Cholesterol (μmol/g)	316.5[a] ±15.1	309.8[a] ±10.3	301[a] ±25.1	244.5[b] ±21.1
Unesterified Cholesterol (μmol/g)	146.6[a] ±8.9	151.5[a] ±5.8	145.9[ab] ±12.7	123.5[b] ±7.7
Triglycerides (μmol/g)	211.7 ±13.8	191.3 ±9.9	212.9 ±8.7	202.7 ±9.0
Liver membranes				
Total Cholesterol (μmol/g)	483[a] ±43.2	512.2[a] ±48.3	440.3[ab] ±61	377.7[b] ±29.8
Unesterified Cholesterol (μmol/g)	252.9[a] ±19	268.8[a] ±12	248.2[a] ±19	199.4[b] ±16
Triglycerides (μmol/g)	142.6 ±10.1	146.0 ±8.0	141 ±3.8	133.0 ±4.4

Values are expressed as mean ± SEM

Values without common superscripts are significantly different, determined using a one way ANOVA and Fishers least significant difference ($p < 0.05$).

There were no significant changes in triglycerides in the homogenate or liver membranes.

($p < 0.05$) (Table 5.6). Unesterified cholesterol constituted approximately 50-55% of the total cholesterol concentration and consumption of the crude catechin extract did not alter this percentage significantly.

There were also significant inverse linear trends between both total and unesterified cholesterol concentrations in the liver membrane fraction and the dose of the crude catechin extract (r = -0.42 and –0.518 respectively, $p < 0.05$). In the liver membrane fraction there were also significant reductions in both total and unesterified cholesterol concentrations (-22% and -21% respectively) in the 2% w/w treatment group compared to the controls ($p < 0.05$). Unesterified cholesterol was approximately 47% of the total cholesterol concentration and remained unchanged with crude catechin consumption. There were no significant trends between triglyceride concentrations in both the homogenate and the liver membrane fraction and the dose of crude catechin extract administered (Table 5.6).

5.3.6. Cholesterol Synthesis and the Intrinsic Capacity to Absorb Dietary Cholesterol

Administration of the crude catechin extract produced a significant inverse linear trend between the ratio of plasma lathosterol to plasma cholesterol, an index of cholesterol synthesis, and the dose of crude catechin extract (r = -0.62, $p < 0.01$). There were significant reductions in cholesterol synthesis in the 1% and 2% (w/w) treatment groups (-50% and –60%) compared to the control ($p < 0.05$) (Figure 5.4). There was no significant trend between the intrinsic capacity to absorb dietary cholesterol by rabbits and the dose of the crude catechin extract: Values were: 7.28 ± 0.64, 7.23 ± 0.64, 8.34 ±

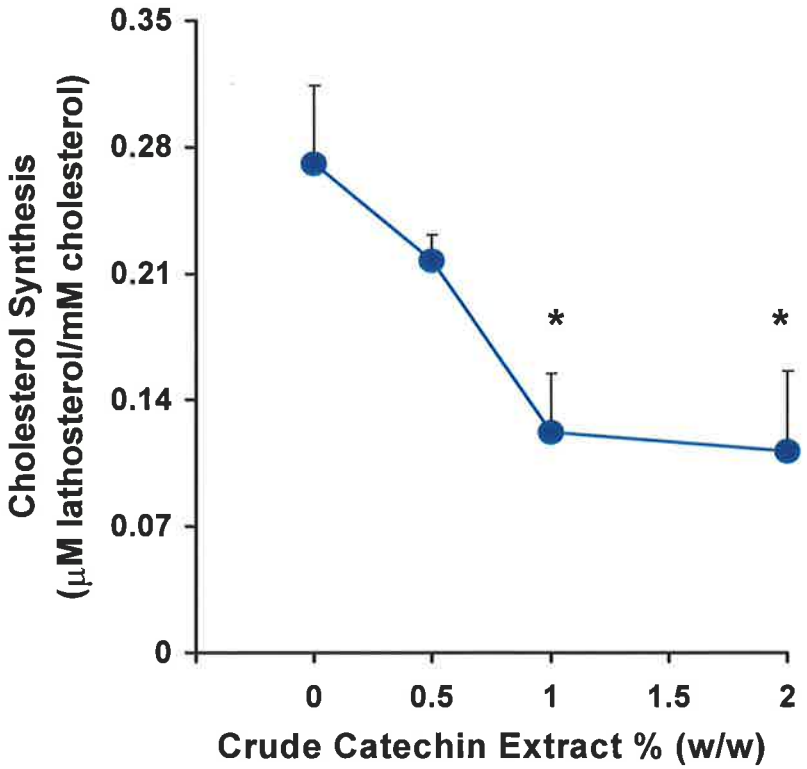


Figure 5.4. Effect of the crude catechin extract from green tea on cholesterol synthesis. Twenty four hypercholesterolaemic rabbits, randomised into 4 treatment groups of 6 rabbits each, were fed a crude catechin extract at concentrations of 0, 0.5, 1 or 2% (w/w) mixed in with normal rabbit chow and 0.25% (w/w) cholesterol for 28 days. The plasma ratios of lathosterol to cholesterol were determined using gas chromatography. Values are expressed as mean ± SEM. (*), significantly different compared to control group using a one way ANOVA and Fishers LSD, Bonferoni and Scheffe post hoc tests of significance ($p<0.05$).

0.55 and 6.42 ± 0.40 (μM phytosterols/mM cholesterol) for the 0, 0.5, 1 and 2% treatment groups respectively.

5.3.7. LDL Receptor

There was a significant positive linear trend between LDL receptor binding activity and the dose of crude catechin extract ($r = 0.58$, $p < 0.01$). The hepatic LDL receptor binding activity was found to be significantly higher in the 2% w/w group (+80%) when compared to the control ($p < 0.05$). A significant positive linear trend was also found between the relative amounts of LDL receptor protein and the dose of the crude catechin extract ($r = 0.45$, $p < 0.05$) and there was a significant increase in the relative amounts of LDL receptor protein in the 2% w/w treatment group (+70%) when compared to the control ($p < 0.05$) (Fig. 5.5).

5.3.8. Correlations

Cholesterol synthesis, measured as the plasma lathosterol to cholesterol ratio, was significantly correlated to plasma total cholesterol ($r=0.578$, $p=0.008$), LDL cholesterol ($r=0.529$, $p=0.016$), VLDL cholesterol ($r=0.560$, $p=0.010$) and unesterified cholesterol in the liver membranes ($r=0.37$, $p=0.05$). Cholesterol synthesis was also weakly positively correlated to total cholesterol in the liver membranes but this did not reach significance ($r=0.35$, $p=0.063$). There was a weak negative relationship between plasma total cholesterol and the hepatic LDL receptor binding activity that did not reach significance ($r=-0.370$, $p=0.080$). Cholesterol synthesis was negatively correlated to LDL receptor binding activity ($r=-0.518$, $p=0.020$) but its relationship with free cholesterol in the liver membrane fraction was not significant ($r=-0.379$, $p=0.100$) (Table 5.7).

Total cholesterol in the thoracic aorta was significantly negatively correlated to plasma total cholesterol concentrations (r=-0.508, p=0.014) and cholesterol synthesis (r=-0.504, p=0.028) (Table 5.7). When comparing the plasma ratios of total cholesterol in VLDLIDL : HDL, LDL : HDL and VLDL + IDL + LDL : HDL fractions to total cholesterol in the thoracic aorta there were significant positive correlations of: r = 0.497 (p=0.020), r=0.464 (0.030) and r=0.500 (p=0.020) respectively.

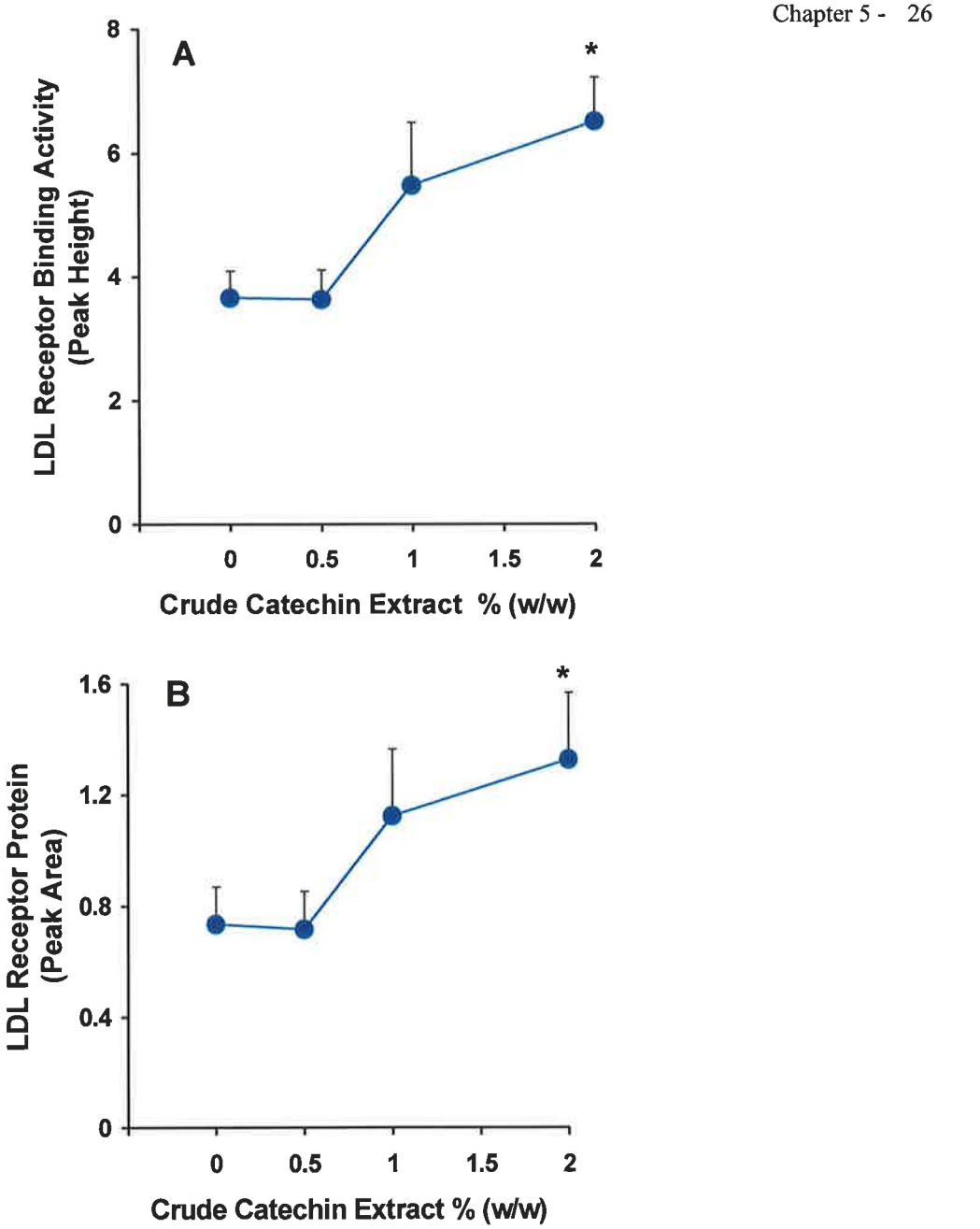
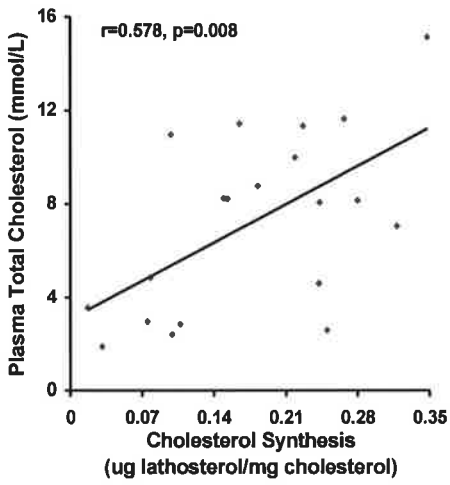


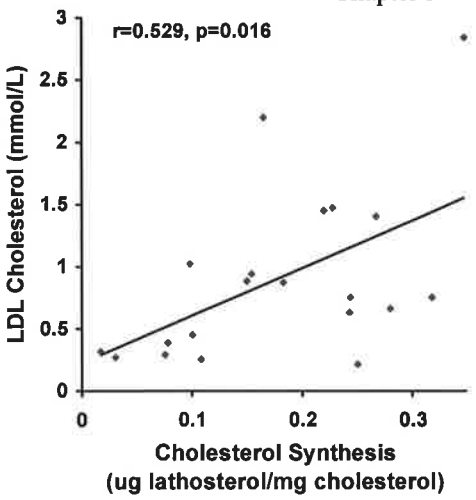
Figure 5.5. Effect of the crude catechin extract from green tea on (A) hepatic LDL receptor binding activity and (B) protein. Twenty four hypercholesterolaemic rabbits, randomised into 4 treatment groups of 6 rabbits each, were fed a crude catechin extract at concentrations of 0, 0.5, 1 or 2% (w/w) mixed in with normal rabbit chow and 0.25% (w/w) cholesterol for 28 days. Hepatic LDL receptor binding activity was determined as the calcium-dependant-binding of LDL-gold to solubilised membrane proteins dot blotted onto nitrocellulose. The relative amounts of hepatic LDL receptor protein were determined using a polyclonal antibody against the hepatic LDL receptor and Western Blotting. Values are expressed as mean ± SEM. (*) denotes a significant difference compared to the control using a one way ANOVA and Fishers LSD ($p < 0.05$).

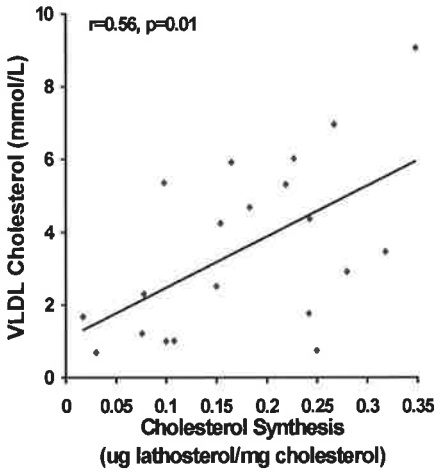
Table 5.7 Correlations between measured parameters

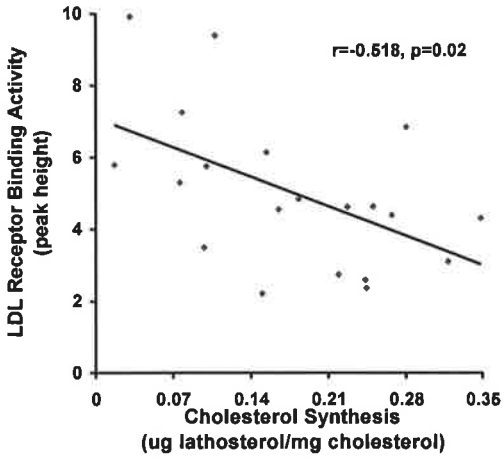
Parameters			*r* value*	*p* value**
Cholesterol Synthesis	vs	Plasma Total Cholesterol	0.578	0.008
Cholesterol Synthesis	vs	LDL Cholesterol	0.529	0.016
Cholesterol Synthesis	vs	VLDL Cholesterol	0.560	0.010
Cholesterol Synthesis	vs	LDL Receptor Binding Activity	-0.518	0.020
Cholesterol Synthesis	vs	Unesterified Cholesterol in Liver Membranes	0.379	0.050
Cholesterol Synthesis	vs	Total Cholesterol in Thoracic Aorta	0.504	0.028
Plasma Total Cholesterol	vs	Total Cholesterol in Thoracic Aorta	0.508	0.014
LDL Receptor Binding Activity	vs	Plasma Total Cholesterol	-0.370	0.080

* r = correlation coefficient, ** $p<0.05$ is significant









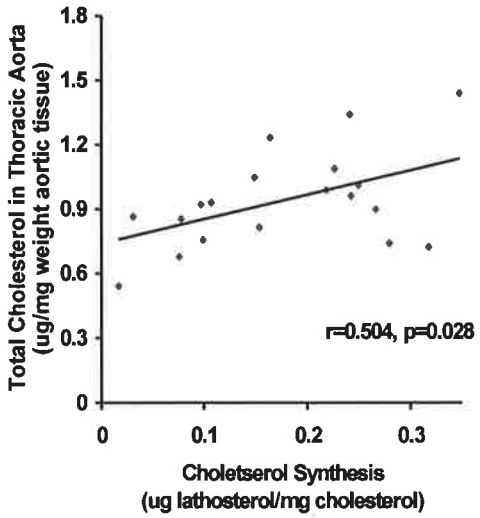


Figure 5.6. The relationship between cholesterol synthesis and other measured parameters.

5.4 Discussion

The present study found that a crude catechin extract from green tea lowered total plasma cholesterol concentrations in the cholesterol-fed hypercholesterolaemic rabbit. It also produced lower cholesterol concentrations in the VLDL + IDL and LDL fractions thereby improving the atherogenic profile. There were however, no changes in the triglyceride or protein concentrations in these lipoprotein fractions, indicating that the number of lipoproteins remained unchanged and these lipoproteins were depleted of cholesterol. There was also a significant decrease in the cholesterol measured in the descending thoracic aorta although there were no changes seen when the aorta was stained for lipid deposition.

Administration of the crude catechin extract also significantly decreased the cholesterol synthesis index and increased the hepatic LDL receptor. These effects provide mechanisms to explain the hypocholesterolaemic effect of the green tea extract used in this study. However, the reduction in cholesterol synthesis appears to be the main driving mechanism because it was more strongly correlated with plasma cholesterol and other parameters of cholesterol metabolism, than was the LDL receptor.

The finding that the crude catechin extract from green tea lowered plasma cholesterol in the cholesterol-fed rabbit is consistent with other animal intervention studies in hamsters, rats and mice (Muramatsu *et al.*, 1986, Matsuda *et al.*, 1986, Fukuyo *et al.*, 1986, Yang and Koo 1997, Chan *et al.*, 1999 and Yang and Koo 2000). This is the first study, however, to demonstrate this effect in the cholesterol-fed hypercholesterolaemic rabbit model. In contrast, Tijburg *et al.* (1997) recently found no changes in plasma cholesterol when they fed a green tea extract to their cholesterol-fed hypercholesterolaemic rabbits.

One difference between the two studies was the way the extract was presented to the rabbits. In the present study, the extract was mixed in the solid diet along with cholesterol while Tijburg et al. (1997) dissolved their green tea extract in water and gave it to the rabbits as their sole drinking source. It may be that the active components in green tea are better absorbed when in the solid diet than in the drinking water. It is also possible that catechins are better able to complex with the cholesterol when they are both in the same medium, thereby preventing their incorporation into mixed bile salt micelles (Ikeda *et al.*, 1992). Another difference between the two studies is the hypercholesterolaemia achieved. The rabbits had an average plasma cholesterol concentration some 4 times higher in Tijburg's study compared to the present study. This is possibly because we initially fed our rabbits the control diet for a shorter period (2 weeks compared with 9 weeks) and it contained a lower fat concentration. The lower starting plasma cholesterol concentration may have made it easier for plasma cholesterol to be lowered by our green tea extract.

The hypocholesterolaemic effects of green tea catechins in animals have been attributed to their ability to inhibit cholesterol absorption from the intestine (Chisaka *et al.*, 1988, Ikeda *et al.*, 1992). In this study, the intrinsic capacity of the intestine to absorb dietary cholesterol, as measured by the plasma ratio of phytosterols to cholesterol (Tilvis and Meittinen, 1986) did not change after the consumption of the crude catechin extract. These results are supported by the observations of Chan *et al.*, (1999) who found that green tea catechins did not alter the activity of intestinal acyl Co A:cholesterol acyltransferase (ACAT), an enzyme which is rate limiting for the intestinal esterification and absorption of cholesterol (Balasbramaniam *et al.*, 1990). The crude catechin extract therefore does not appear to inhibit cholesterol absorption by decreasing the intrinsic

capacity of the intestine to absorb the sterol. As proposed by Ikeda *et al*, (1992), the catechins are more likely to inhibit cholesterol absorption by interfering with the biliary micelle system in the lumen of the intestine.

The upregulation of the LDL receptor observed in this study is consistent with the increased faecal excretion of cholesterol and bile acids found in other animal studies (Chan *et al.*, 1999, Yang and Koo 2000). This is because the LDL receptor is normally increased in response to a reduction in cholesterol absorption to increase the influx of cholesterol into cells. The upregulation of the hepatic LDL receptor in the present study by the crude catechin extract is also consistent with the increase in the LDL receptor, observed *in vitro* in HepG2 cells after treatment with freshly brewed green tea (Chapter 3) and EGCG (Chapter 4). This indicated that the effects seen *in vitro* on the LDL receptor could be translated *in vivo* in the hypercholesterolaemic rabbit. An increase in the LDL receptor is one mechanism by which the crude catechin extract could lower plasma cholesterol concentrations. It is, however, only weakly correlated with plasma cholesterol in this study ($r = -0.37$, $p = 0.08$).

The decrease in cholesterol synthesis, however, is not consistent with an increase in the faecal excretion of cholesterol and bile acids. It appears to be a separate, systemic effect of the crude catechin extract where it is acting directly to inhibit cholesterol synthesis. This is possible because catechins are known to be absorbed into the circulation and can be measured in the plasma and the tissues (Nakagawa *et al.*, 1997, Suganuma *et al.*, 1998). The reduction in cholesterol synthesis in the present study is, however, consistent with the reduction in cholesterol synthesis observed *in vitro* in HepG2 cells after

treatment with the lower doses of freshly brewed green tea (Chapter 3) and EGCG (Chapter 4).

Furthermore, this decrease in cholesterol synthesis appears to be the initiating mechanism by which the crude catechin extract produces its cholesterol lowering effects rather than the increase in the LDL receptor. This is suggested by the strong correlation between cholesterol synthesis and plasma cholesterol. Cholesterol synthesis is also strongly correlated to LDL cholesterol, VLDL + IDL cholesterol as well as cholesterol in the thoracic aorta (Table 5.7). In addition to this, there is a significant negative correlation between cholesterol synthesis and hepatic LDL receptor binding activity. This may suggest a possible mechanism for the increase in the LDL receptor whereby the decreased level of cholesterol synthesis may reduce the size of the "active pool" of cholesterol and hence the formation of oxysterol, which are thought to regulate the LDL receptor. A decrease in oxysterol concentrations will increase the activation of SREBPs, transcription factors for the LDL receptor. These effects of the crude catechin extract are similar to hypocholesterolaemic drugs such as lovastin, simvastatin and pravastatin, which reduce cholesterol synthesis and increase the LDL receptor (Roach *et al.*, 1993, Parker *et al.*, 1990, Reihner *et al.*, 1990, Endo 1992).

The way in which cholesterol synthesis is lowered by green tea and its catechins is likely to be due to an inhibition in HMGCoA reductase and this may be a result of its antioxidant properties. A study by Feurgard *et al.*, 1999 found that when hamsters were radiated with sub-lethal whole-body Co60 gamma-irradiation, there was a significant increase in HMGCoA reductase activity. Furthermore, a study by Gesquiere *et al.* (1999) found that oxidative stress, initiated by using a free radical generator called azobis-

amidinoproprane dihydrochloride, also significantly increased HMGCoA reductase activity. It appears, therefore, that this enzyme can be regulated through free radical modifications, where an increase in oxidative stress can increase its activity. Green tea and catechins possess potent free radical scavenges activity *in vitro* and *in vivo* therefore can reduce oxidative stress (Section 1.11.3). Taken together, these observations in the literature provide a possible mechanism by which green tea and its catechins can reduce cholesterol synthesis, where their antioxidant activities reduce the oxidative stress of their environment and reduce HMGCoA reductase activity.

It is acknowledged that there was large variability within treatment groups after the consumption of the crude catechin extract, therefore, the statistically significant differences achieved in cholesterol metabolism compared to control values may have been due to chance. In animal studies of any kind there is usually a large variability within treatment groups as some animals will respond to treatments in different ways. Despite this, however, in this study there were substantial changes in the main parameters of cholesterol metabolism such as plasma and LDL cholesterol concentrations (-60%, -80% respectively), cholesterol synthesis (-60%) and LDL receptor binding activity and protein (+80%, +70% respectively). There were also strong correlations between these various parameters (Table 5.7). This therefore indicates that the changes witnessed in these rabbits after the consumption of the crude catechin extract were in fact biologically significant and not due to chance.

In conclusion this study found that the administration of a crude catechin extract from green tea could reduce plasma, liver and thoracic aorta cholesterol in the cholesterol-fed hypercholesterolaemic rabbit. The crude catechin extract also reduced cholesterol

synthesis and increased the LDL receptor. These findings are consistent with the *in vitro* studies in HepG2 cells where freshly brewed green tea (Chapter 3) and EGCG (Chapter 4) reduced cholesterol synthesis in the lower dose treatment groups and increased the LDL receptor. The effects seen *in vitro* on cholesterol metabolism were therefore translated *in vivo* in the hypercholesterolaemic rabbit. The reduction in cholesterol synthesis and the increase in the LDL receptor most likely contributed to the lowering of the plasma cholesterol. This provides mechanisms to explain the hypocholesterolaemic effects of green tea extracts and purified catechins in epidemiological and animal studies. The reduction in cholesterol synthesis appears to be more relevant however, as it is the parameter most highly correlated with plasma cholesterol concentrations.

Chapter 6

General Discussion

GENERAL DISCUSSION

The main objectives of the present study were 1) to determine the mechanism by which green tea and EGCG increased the LDL receptor in HepG2 cells and 2) to investigate weather a crude catechin extract from green tea could lower plasma cholesterol levels in the hypercholesterolaemic rabbit and ascertain if this effect was due to an increase in the LDL receptor.

6.1 Mechanisms by which Freshly Brewed Green Tea and EGCG Modulated Cholesterol Metabolism in the HepG2 Cells.

Consistent with our previous studies (Bursill 1996, Pal *et al.*, 1999), the present study found that freshly brewed green tea and purified EGCG increased LDL receptor binding activity and protein levels in HepG2 cells. The mechanism for this effect appears to be mediated by an increase in the conversion of SREBP-1c from its inactive precursor form to its active mature form. The mature active form of SREBP-1c is a transcription factor for the LDL receptor which binds upstream of its promoter region to increase transcription. In addition to this both green tea and EGCG were able to significantly reduce cellular total cholesterol concentrations, produce a biphasic "down-then-up" effect on cholesterol synthesis and appeared to increase cholesterol efflux in the highest dose treatment group. This indicates that EGCG may be the active ingredient in green tea because the effects of EGCG on cholesterol metabolism in HepG2 cells were very similar to those of freshly brewed green tea.

Although there was a consistent decrease in cellular cholesterol and a consistent increase in the LDL receptor at all concentrations of freshly brewed green tea and EGCG tested, it appears that the mechanism by which these changes occurred were different depending on the treatment concentration. This was indicated by the differences observed for cholesterol synthesis at the lower dose compared to the higher dose of green tea and EGCG. Cholesterol synthesis was decreased with the lower dose and increased with the higher dose of both freshly brewed green tea and EGCG.

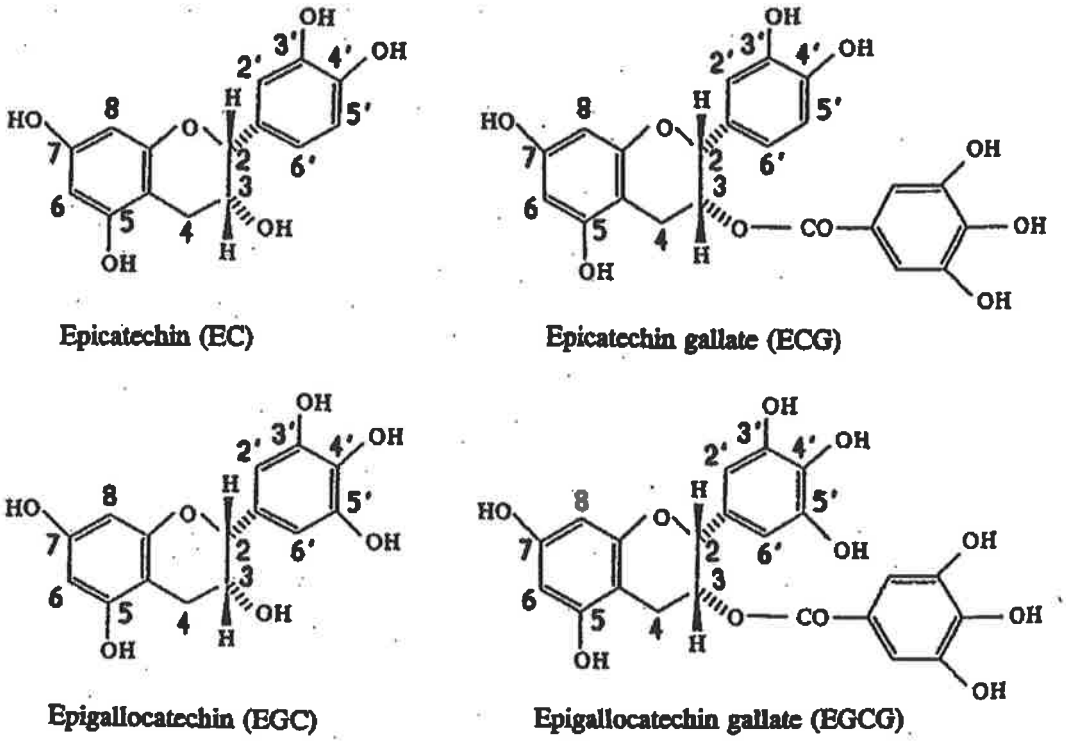
6.1.1 Lower dose treatments (Figure 6.2, Scheme A)

At the lower treatment concentrations (Figure 6.2, Scheme A), the decrease in cellular cholesterol and the increase in LDL receptor appeared to be due to a decrease in cholesterol synthesis (Chapters 3 and 4). The decrease in cholesterol synthesis may have reduced the size of the "active pool" of unesterified cholesterol, as less cholesterol was being produced. If the size of the "active pool" of unesterified cholesterol was reduced then, less oxysterol may have been formed, thereby increasing the activation of SREBP-1c as found with the higher dose concentrations (Chapters 3 and 4) and subsequently upregulating the LDL receptor.

The effects of freshly brewed green tea and EGCG at the lower dose are similar to the effects of hypocholesterolaemic drugs of the statin class (e.g. lovastin, simvastatin and pravastatin). These drugs are well known to reduce cholesterol synthesis and often lead to an increase the LDL receptor (Roach *et al.*, 1993, Parker *et al.*, 1990, Reihner *et al.*,

1990, Endo 1992). The statins have large complex chemical structures with multiple benzene rings surrounded by hydroxyl and methyl groups, which could be likened to the chemical structures of the catechins (Figure 6.1). It may therefore be their structural similarities that produce their similar effects on cholesterol metabolism. Mevastatin, for example, has been clearly shown to inhibit cholesterol synthesis by competing with the HMGCoA reductase substrate and this is thought to be due to its structural similarity to HMGCoA (Endo 1992). The catechins may perhaps compete with HMGCoA in the same fashion to decrease cholesterol synthesis as observed with the lower dose treatments of HepG2 cells (Chapters 3 and 4).

Alternatively, the antioxidant properties of green tea and its catechins may be responsible for a decrease in HMGCoA reductase activity. A study by Feurgard *et al.*, 1999 found that when hamsters were radiated with sub-lethal whole-body Co60 gamma-irradiation, there was a significant increase in HMGCoA reductase activity. Furthermore, a study by Gesquiere *et al.* (1999) found that oxidative stress, initiated by using a free radical generator called azobis-amidinoproprane dihydrochloride (AAPH), also significantly increased HMGCoA reductase activity. It appears, therefore, that this enzyme is "redox sensitive" and may be regulated through free radical oxidative mechanisms, either directly or indirectly through other modulators such as cAMP as suggested by Gesquiere *et al.* (1999). Green tea and catechins possess potent free radical scavenging activity *in vitro* and *in vivo* and can therefore reduce oxidative stress (Section 1.11.3). If an increase in oxidative stress can increase HMGCoA reductase activity, green tea and its catechins may



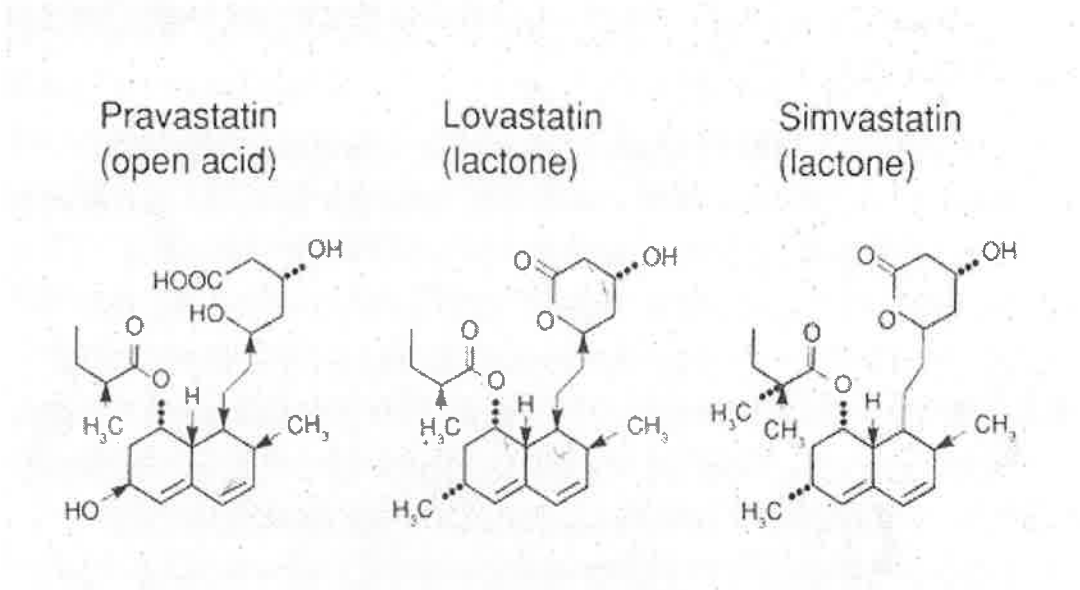


Figure 6.1 Similarities in the chemical structures of (A) the catechins and (B) the statins

thereby reduce cholesterol synthesis, by reducing the oxidative stress of their environment and reduce HMGCoA reductase activity.

Although it has been hypothesised above that green tea and EGCG decrease cholesterol synthesis by reducing HMGCoA reductase activity, the activity was measured using the "in vitro" assay (Gesquiere *et al.*, 1999, Feurgard *et al.*, 1999), which, as mentioned previously, does not always reflect the level of cholesterol synthesis (Section 1.12.3). In addition to this, animal intervention studies in hamsters and rats have found no effect on the "in vitro" activity of HMGCoA reductase (Chan *et al.*, 1999, Yang and Koo 2000). However, if green tea and EGCG do not lower cholesterol synthesis by reducing HMGCoA reductase activity, they may affect one or more of the many other enzymes involved further downstream in the cholesterol biosynthetic pathway.

SCHEME A

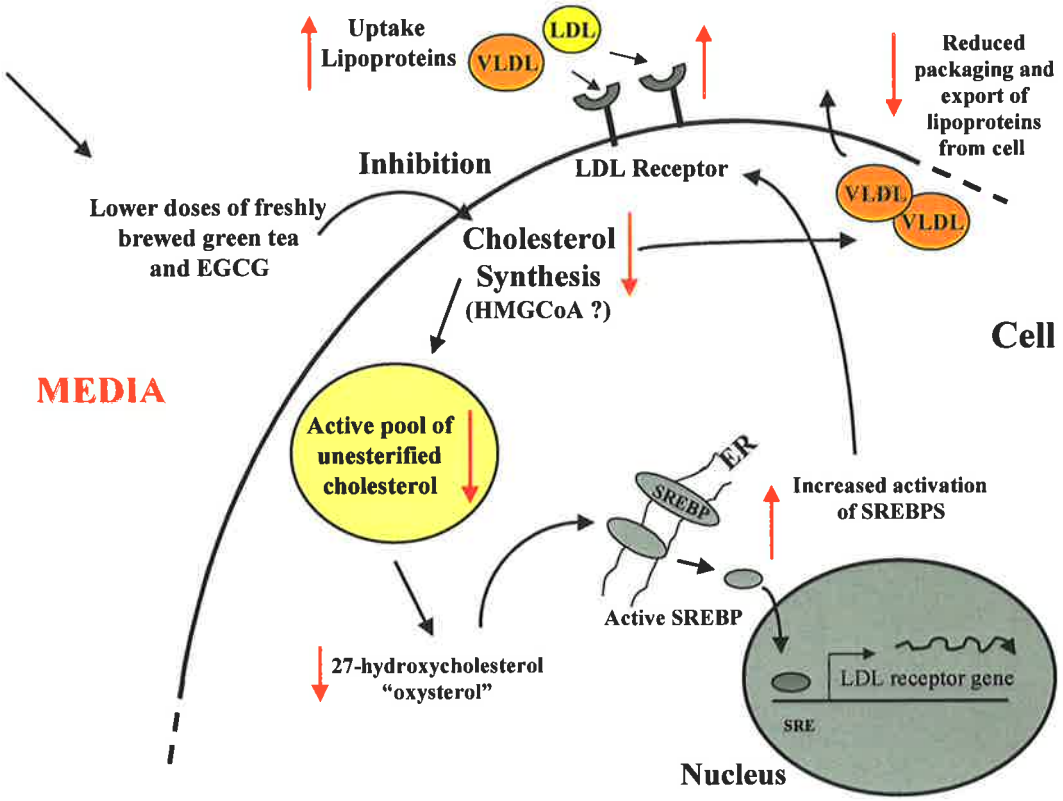


Figure 6.2. Diagrammatic representation of the effects of freshly brewed green tea and EGCG on cholesterol metabolism in HepG2 cells with the lower doses. Cellular cholesterol concentrations are decreased and the LDL receptor is upregulated in response to a reduction in cholesterol synthesis.

6.1.2 Higher dose treatments (Figure 6.3, Scheme B)

Treatment with freshly brewed green tea and EGCG significantly decreased cellular cholesterol and upregulated the LDL receptor at the higher dose and this was associated with an increase in the activation in SREBP-1c (Chapters 3 and 4). In contrast to the lower dose treatments however, the effects at the higher dose were not due to a decrease in cholesterol synthesis as it was actually significantly increased (Chapters 3 and 4). There was also a significant increase in media cholesterol concentrations (Chapters 3 and 4), most likely due to an increased efflux of cholesterol from the cells.

A study by Ikeda *et al.*, (1992) found that EGCG was able to form insoluble complexes with cholesterol and this may explain what happened with the higher dose treatment. At this dose, the concentration of EGCG may have been great enough to complex with enough cholesterol in the media to render it essentially cholesterol deficient, with the cells not being able to use the cholesterol that was complexed with EGCG. The cholesterol may then have moved from the cells to the media by normal diffusion down a concentration gradient, thereby increasing the efflux of cholesterol from the cells to the media. This loss of cholesterol from the cell may have lead to a reduction in the "active pool" of unesterified cholesterol, leading to a decrease in the formation of oxysterols. A decrease in oxysterol formation could explain the increased activation of SREBP-1c and the subsequent increase in the LDL receptor. This scenario (Figure 6.3, Scheme B) is also consistent with the observed increase in cholesterol synthesis as SREBP-1c also regulates the HMGCoA reductase gene, the rate-limiting enzyme in cholesterol synthesis.

SCHEME B

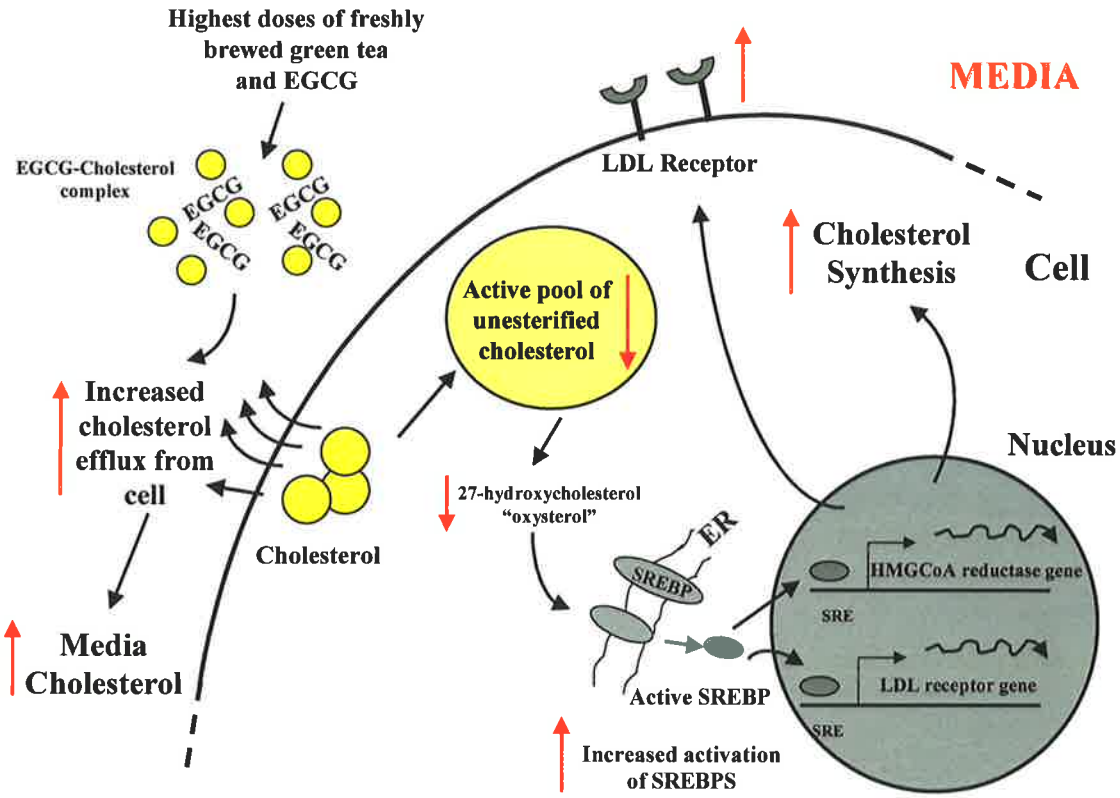


Figure 6.3. Diagrammatic representation of the effects of freshly brewed green tea and EGCG on cholesterol metabolism in HepG2 cells with the higher doses. Cellular cholesterol concentrations are decreased and the LDL receptor is upregulated in response to an increased cholesterol efflux from the cells. These effects appear to override any inhibitory effects that green tea and EGCG may have on cholesterol synthesis as it is significantly increased in order to compensate for the loss of cell cholesterol.

6.2 Mechanism by which the Crude Catechin Extract Modulated Cholesterol Metabolism in the Rabbits.

To determine whether the effects seen *in vitro* with HepG2 cells, were translated *in vivo*, rabbits were administered a crude catechin extract from green tea. This treatment decreased plasma, VLDL + IDL and LDL cholesterol concentrations. There was also an increase in the LDL receptor and a decrease in cholesterol synthesis (Chapter 5). The increase in the LDL receptor is consistent with *the vitro* studies at both the lower (Scheme A) and higher (Scheme B) dose treatments. However, it appeared that the effects of the crude catechin extract on cholesterol metabolism in the rabbits (Scheme C, Figure 6.4) was most like the effects seen with the lower doses of green tea and EGCG (Scheme A). As with Scheme A, there was a significant decrease in cholesterol synthesis and it appears that the reduction in cholesterol synthesis was responsible for the increase that occurred in the hepatic LDL receptor in the rabbits. As mentioned above, the decrease in cholesterol synthesis could have reduced the "active pool" of unesterified cholesterol, which in turn would have decreased the formation of oxysterols. A decrease in the oxysterol concentration could then increase the activation of SREBPs and hence the LDL receptor.

SCHEME C

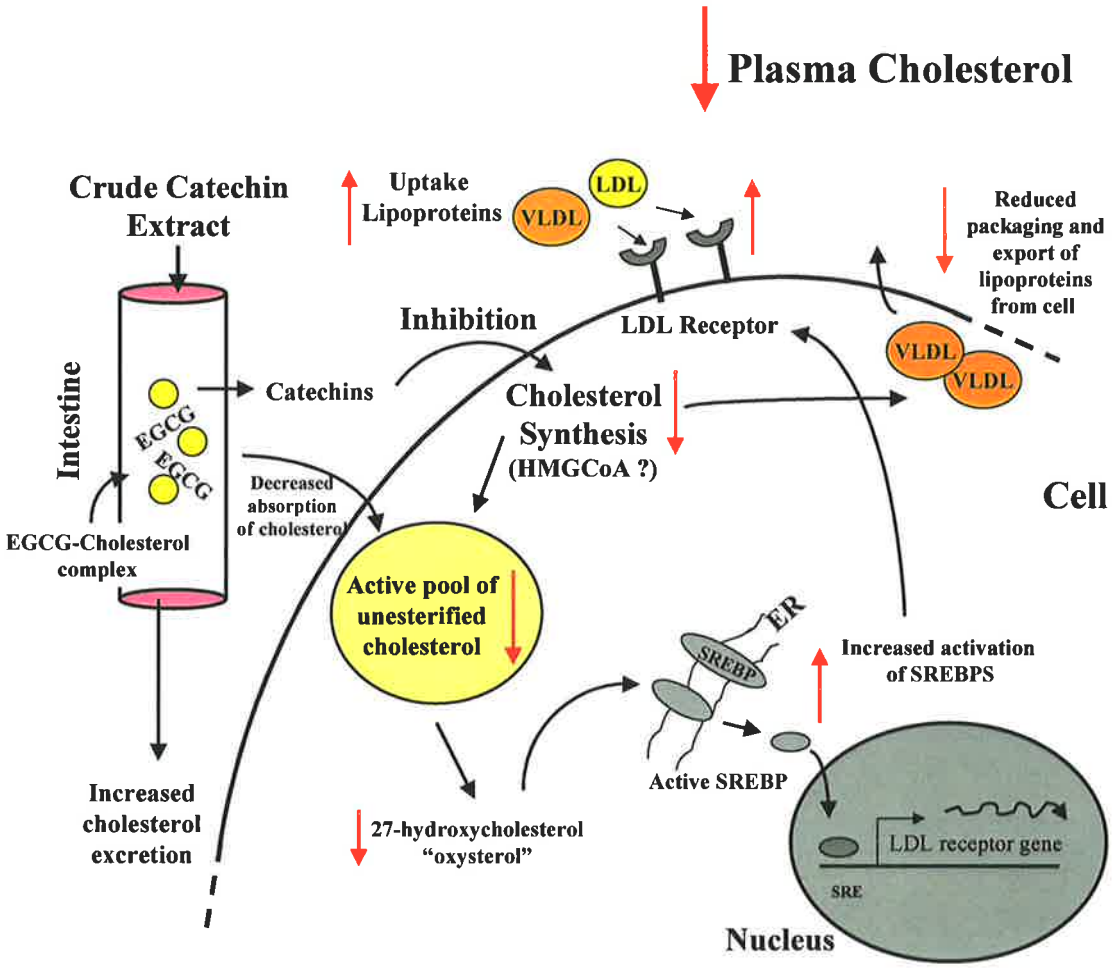


Figure 6.4. Diagrammatic representation of the effects of the crude catechin extract on cholesterol metabolism in the rabbit. It appears that the cholesterol-lowering effects of the crude catechin extract were due to a decrease in cholesterol synthesis and an increase in the LDL receptor. Catechins, according to the literature, also inhibit cholesterol absorption. These effects of the crude catechin extract on cholesterol metabolism are similar to the effects observed with HepG2 cells at the lower doses of freshly brewed green tea and EGCG.

It may be expected that the effects of the crude catechin extract on cholesterol metabolism in rabbits (Scheme C) were most similar to the effects of freshly brewed green tea and EGCG on cholesterol metabolism in HepG2 cells at the lower doses (Scheme C). This is not suprising because catechins are poorly absorbed into the circulation (2% maximum of that ingested, Section 1.11.2). For example, a study by Nakagawa *et al.*, (1997) found that one hour after administration of EGCG, the concentration of EGCG in the plasma peaked at 12.3 μM and at 48.4 nmol/mg in the liver. As it is therefore unlikely that the plasma and liver concentrations of catechins could reach the same levels as those in the higher doses of green tea (200 μl in 10 ml media) or EGCG (200 μM) used with the HepG2 cells, it may be expected that their effects on cholesterol metabolism in rabbits would be different from those seen with HepG2 cells in the higher doses. The crude catechin extract therefore affected cholesterol metabolism in a fashion more similar to the lower dose treatments (Scheme A). Catechin concentrations are also transient; after administration their plasma concentrations peak within 1-2 hour and then decrease to undetectable levels within 8 hours. Therefore after an overnight fast, as when blood samples were taken in the present rabbit study (Chapter 5), catechin concentrations would be too low to be detected and consequently were not measured.

The hypocholesterolaemic effects of green tea extracts have been attributed to their ability to inhibit cholesterol absorption. Support for this has come from studies that have found the administration of green tea extracts to animals increased the faecal excretion of total lipids and cholesterol (Muramatsu *et al.*, 1986, Fukuyo *et al.*, 1986, Matsuda *et al.*,

1986, Chan *et al.*, 1999). EGCG has also been observed to inhibit the uptake of ^{14}C-cholesterol from the intestine (Chisaka *et al.*, 1988).

When there is a reduction in cholesterol absorption, it is usually accompanied by an increase in both the LDL receptor and cholesterol synthesis. These effects have been noted with inhibitors of cholesterol synthesis such as tiqueside (Harwood *et al.*, 1993) and inhibitors of intestinal reabsorption of bile acids such as cholestyramine (Dory *et al.*, 1990, Rudling 1992). The LDL receptor increases to enhance the uptake of LDL cholesterol from the circulation and cholesterol synthesis increases to produce more cholesterol in order to compensate for the reduced entry of cholesterol into the circulation (Brown and Goldstein 1986).

In the present study, there was a significant increase in the hepatic LDL receptor in rabbits This is consistent with a reduction in cholesterol absorption. It is also consistent with the increase in the LDL receptor that occurred in HepG2 cells after treatment with green tea and EGCG. Not consistent with a reduction in cholesterol absorption, however, is the significant decrease in cholesterol synthesis, which occurred after the administration of the crude catechin extract to rabbits. This therefore appears to be a separate, systemic effect of the crude catechin extract where it is acting directly to inhibit cholesterol synthesis. This is possible because catechins are known to be absorbed into the circulation and can be measured in the plasma and the tissues (Nakagawa *et al.*, 1997, Suganuma *et al.*, 1998). As mentioned above, the catechins have poor bioavailability and therefore only low concentrations must be required to produce their inhibitory effects on cholesterol synthesis.

Furthermore, this effect of the catechins on cholesterol synthesis must override any possible increase in synthesis that may occur as a result of a reduction in cholesterol absorption.

A decrease in cholesterol synthesis and an increase in the LDL receptor as found in the rabbit study (Chapter 5) are two plausible mechanisms for explaining the hypocholesterolaemic effects of green tea extracts and purified catechins found in epidemiological and animal intervention studies. The reduction in cholesterol synthesis, however, appears to be the main driving force behind these cholesterol-lowering effects as it was more strongly correlated with plasma cholesterol concentrations and other parameters of cholesterol metabolism, compared to the increase in the LDL receptor. Just as the effects of green tea and EGCG at the lower doses with HepG2 cells (Scheme A), were similar to the hypocholesterolaemic effects of drugs of the statin class, so too were the effects of the crude catechin extract in the rabbits. In addition to this, the reductions in plasma cholesterol with the crude catechin extract (-60%) were comparable to those attained after statin treatments. For example, a study by Roach *et al.*, (1993) found that simvastatin and pravastatin reduced plasma cholesterol by 55% compared to the control in the cholesterol-fed rabbit. This is comparible to the 60% reduction in the present study.

As mentioned previously, the structural similarities between catechins and the statins may explain their similar effects on cholesterol metabolism. If catechins compete with HMGCoA in the same fashion as the statins it may provide a mechansim to explain the decrease in cholesterol synthesis. Alternatively, the catechins may lower cholesterol

synthesis by decreasing HMGCoA reductase activity as a result of their antioxidant properties (Section 5.4 and 6.1.1). If catechins do not, however, lower cholesterol synthesis by reducing HMGCoA reductase activity they may then act to inhibit enzymes further down the cholesterol biosynthetic pathway.

The ability of green tea and its catechins to act in the same way as the potent plasma cholesterol-lowering statins (ie lowering cholesterol synthesis and increasing the LDL receptor), in combination with their ability to inhibit cholesterol absorption (Muramatsu *et al.*, 1986, Matsuda *et al.*, 1986), indicates that they could potentially be very potent cholesterol-lowering agents. Despite this however, current human intervention studies have failed to find that green tea lowers plasma cholesterol concentrations (van het Hof *et al.* 1997, Princen *et al.*, 1998). One possible reason for this lack of hypercholesterolaemic effect may be that the 4 week treatment period, used in these studies, was not sufficient to produce a change in plasma cholesterol concentrations. In addition to this, the subjects in these studies were normolipidaemic and therefore a reduction in plasma cholesterol concentrations may have been harder to achieve. It is also possible that the dose of green tea administered was not great enough (6 cups/day), particularly as the bioavailability of the catechins is poor. The bioavailability of the catechins may be improved, however, if a crude catechin extract was incorporated into the solid diet in a similar fashion to the present study (Chapter 5).

In summary, this study demonstrates that green tea and its catechins can modify cholesterol metabolism *in vitro* and *in vivo* and helps to elucidate the possible mechanisms

involved. The LDL receptor was increased in both HepG2 cells and in the hypercholesterolaemic rabbit. Cholesterol synthesis was affected differently in HepG2 cells depending on the treatment concentration. At the lower dose of green tea and EGCG (Scheme A), cholesterol synthesis was lower whilst at the higher doses synthesis was significantly increased (Scheme B). In rabbits the administration of the crude catechin extract significantly decreased cholesterol synthesis (Scheme C), similar to the effects observed with the lower dose treatments in the HepG2 cell studies. This is perhaps to be expected because catechins have a poor bioavailability and therefore plasma catechin concentrations are unlikely to achieve the same levels as those in the higher doses used with the HepG2 cells. Nonetheless, an increase in the LDL receptor and a reduction in cholesterol synthesis are two mechanisms that can explain the cholesterol-lowering properties of the crude catechin extract in the rabbit. A combination of evidence from the epidemiological and the animal intervention studies as well as the work from the present study indicate that green tea and its catechins possess cholesterol-lowering properties and may therefore be protective agents against CHD. However, until a human intervention study is conducted, where a green tea extract successfully lowers plasma cholesterol then the relevance for this effect in humans remains unproven.

6.3. Future Studies

The work herein has provided some interesting and novel findings. There are, however, several questions that could still be addressed. Of interest, is the exact mechanism by which green tea and its catechins reduce cholesterol synthesis. It has been hypothesised in

this study that perhaps it is due to an inhibition of HMGCoA reductase as it is the rate limiting enzyme in cholesterol synthesis. However, we can not be sure exactly at what step in the biosynthetic pathway green tea and its catechin may be acting. It is unlikely that HMGCoA reductase is inhibited at the level of gene transcription because studies in our laboratory have found that a green tea extract, enriched in catechins, significantly increased HMGCoA reductase mRNA in HepG2 cells at all concentrations tested (10-100 μM, Pal *et al.*, 1999). However, an increase in HMGCoA reductase mRNA is often observed in response to an inhibition of the enzyme. Simvastatin, for example, will cause cells to synthesise more HMGCoA reductase mRNA in an attempt to overcome the inhibition at the enzyme level.

Another experiment would be to determine if green tea and its catechins could increase the activation of SREBP-1 and –2 in the rabbit. It was found in this study that SREBP-1c activation was increased in HepG2 cells. Measurement of SREBP-1c and –2 was attempted in the rabbit during this project but was, however, unsuccessful. Unfortunately the commercially available primary antibody is not recommended as reactive towards the rabbit SREBPs. In the rabbit it would be important to determine the effect of green tea and its catechins on both SREBP-1 and -2 because it has been found that the genes of these two proteins are independently regulated in animal models (Section 1.9.3). This is in contrast to cell culture systems where SREBP-1 and –2 appear to be regulated in parallel. An increased activation of SREBPs would provide a mechanism to explain the increase in the hepatic LDL receptor observed in rabbits in this study.

Given the apparent mechanism of action observed in the rabbit, it could be predicted that green tea and its catechins have potent cholesterol-lowering effects. However, this can not be proven without the support of a successful human intervention study. In future human intervention trials, subjects should perhaps be administered a green tea extract, enriched in catechins, mixed in with their solid diet. If this extract is taken in a solid form perhaps catechin absorption will be improved. Catechins may also be better able to complex with the cholesterol if they are taken in the same medium as the cholesterol. Furthermore, if catechins were taken during the main meals, it may allow them to work most efficiently, as they would be able to complex with the cholesterol in those meals. Finally, previous human intervention studies have used a treatment period of 4 weeks. If this was extended to as long as 3 months it would probably increase the likelihood of producing significantly lower plasma cholesterol concentrations.

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