DIETARY COMBINATION OF MINERAL NUTRIENTS AND NATURAL PLANT STEROLS

Effects on Serum Lipids and Blood Pressure in Experimental Obesity

Timo Vaskonen

Institute of Biomedicine
Pharmacology
University of Helsinki

Academic Dissertation

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SUPERVISORS

Professor Heikki Karppanen, MD, PhD
Institute of Biomedicine, Pharmacology
University of Helsinki
Helsinki, Finland

Docent Eero Mervaala, MD, PhD
Institute of Biomedicine, Pharmacology
University of Helsinki
Helsinki, Finland

REVIEWERS

Professor Heikki Ruskoaho, MD, PhD
Department of Pharmacology and Toxicology
University of Oulu
Oulu, Finland

Docent Ilkka Pörsti, MD, PhD
Medical School
Departments of Internal Medicine and Pharmacology
University of Tampere
Tampere, Finland

OPPONENT

Professor Matti Tikkanen, MD, PhD
Helsinki University Central Hospital
Department of Medicine
Helsinki, Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original articles. Some previously unpublished data are also presented. The articles are referred to in the text by their Roman numerals.

I Vaskonen T, Mervaala E, Seppänen-Laakso T, Karppanen H. Diet enrichment with calcium and magnesium enhances the cholesterol-lowering effect of plant sterols in obese Zucker rats. *Nutr Metab Cardiovasc Dis* 11: 158-167, 2001.

II Vaskonen T, Mervaala E, Sumuvuori V, Seppänen-Laakso T, Karppanen H. Effects of calcium and plant sterols on serum lipids in obese Zucker rats on a low-fat diet. *Br J Nutr* 87: 239-245, 2002.

III Vaskonen T, Mervaala E, Seppänen-Laakso T, Laakso I, Karppanen H. Effects of calcium and plant sterols on serum lipids in obese Zucker rats on a high-fat diet. Submitted.

IV Vaskonen T, Mervaala E, Krogerus L, Karppanen H. Supplementation of plant sterols and minerals benefits obese Zucker rats fed an atherogenic diet. *J Nutr* 132: 231-237, 2002.

ABBREVIATIONS

ABCA	ATP-binding cassette
ACAT	Acyl-CoenzymeA:cholesterol acyl-transferace
ACE	Angiotensin converting enzyme
AHA	American Heart Association
ANOVA	Analysis of variance
Apo	Apolipoprotein
ARIC	Atherosclerosis Risk in Communities
ATP	Adenosine triphosphate
C	Cholesterol
CoA	Coenzyme A
DASH	Dietary Approaches to Stop Hypertension
ECF	Extracellular fluid
FAS	Fatty acid synthase
HC	Hypercholesterolemia
HDL	High density lipoprotein
HDL-C	High density lipoprotein cholesterol
LDL	Low density lipoprotein
LDL-C	Low density lipoprotein cholesterol
LVH	Left ventricular hypertrophy
NHANES	National Health and Nutrition Examination Survey
NIH	National Institutes of Health
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
PTH	Parathyroid hormone
RDA	Recommended dietary allowance
SAFA	Saturated fatty acids
SR	Scavenger receptor
TC	Total cholesterol
TG	Triglycerides
VLDL	Very low density lipoprotein
VLDL-C	Very low density lipoprotein cholesterol
w/w	Percentage of weight

ABSTRACT

High serum cholesterol, hypertension and obesity are major risk factors for cardiovascular diseases. Plant sterols and their derivatives have been shown to lower serum cholesterol in patients with mild hypercholesterolemia. Mineral nutrients, such as calcium, potassium and magnesium, lower blood pressure, and especially calcium may also have beneficial effects on serum lipids. The present series of studies was carried out to investigate the effects of a combination of dietary plant sterols and mineral nutrients on serum lipids and blood pressure in obese Zucker rats.

During a high-fat, high-cholesterol diet, a combination of natural plant sterols and the mineral nutrients calcium, magnesium and potassium lowered serum cholesterol levels more effectively than the plant sterols or the minerals alone. This enhancement effect was confined to calcium and magnesium, whereas sodium and potassium had no independent effects on serum cholesterol. Increased intake of dietary calcium and magnesium, both with and without potassium, also reduced the development of obesity during the four to seven week experiments.

Calcium supplementation alone dose-dependently lowered serum total- and LDL cholesterols and raised the HDL to LDL cholesterol ratio during both low- and high-fat diets. Measurements of serum plant sterols and the cholesterol precursors desmosterol and lathosterol suggested that, during the low-fat diet, both intestinal absorption and endogenous synthesis of cholesterol were dose-dependently increased by dietary calcium supplementation. During the high-fat diet, only the synthesis of cholesterol appeared to increase. Plant sterols were particularly useful during low intake of calcium, and their beneficial effects on serum lipids were further enhanced by even a moderate increase in dietary calcium intake. High calcium intake also markedly reduced the development of obesity during a high-fat diet, irrespective of the addition of plant sterols.

In a long-term experiment, fortification of normal rat food with butter, cholesterol and common salt resulted in severe hyperlipidemia, hypertension, and death in 12 months, apparently due to heart infarctions and kidney damage. Addition of plant sterols and replacing the sodium chloride in this atherogenic diet partially with calcium, magnesium and potassium salts effectively prevented the diet-induced increases in total and LDL cholesterols and 24-hour systolic and mean blood pressures, and markedly improved endothelium-mediated vasorelaxation. The combination of plant sterols and minerals also protected against cardiovascular and renal damage and considerably extended the life span of the rats.

Our findings indicate that human-type cardiovascular disorders can be induced in obese Zucker rats by feeding them a human-type atherogenic diet. Supplementing this diet with the combination of mineral nutrients and plant sterols exerts several beneficial effects that are not likely to be achieved by plant sterols alone, including the control of hypertension and obesity. In particular, dietary calcium intake appears to be an important regulator of serum cholesterol levels in obese Zucker rats. The effects of calcium are dose-dependent and resemble those of bile-acid binding resins. With higher doses and during high-fat diets, calcium is also likely to reduce the absorption of saturated fatty acids.

1 INTRODUCTION

Despite very impressive recent advances in coronary risk factor identification and modification, heart attack remains the most common cause of death in Western societies. Major risk or even causative factors for heart attack, as well as other cardiovascular diseases, are elevated blood pressure and serum cholesterol levels and obesity (Stamler et al. 1993; Verschuren et al. 1995; Must et al. 1999). These disorders are often associated with insulin resistance and other disturbances in carbohydrate metabolism; together they comprise the notorious metabolic syndrome, also referred to as syndrome X or Reaven syndrome (Reaven 1993), and multiply the risk of cardiovascular complications and death (DeFronzo & Ferrannini 1991). Clinically, the definition of the metabolic syndrome is somewhat controversial, but according to one of the most recent guidelines (Adult Treatment Panel III 2001), the diagnosis can be made when three or more of the risk determinants shown in TABLE 1 are present.

Table 1. The metabolic syndrome

RISK FACTOR	DEFINING LEVEL
Abdominal obesity	(waist circumference)
Men	> 40 in (102 cm)
Women	> 35 in (88 cm)
Hypertriglyceridemia	≥ 150 mg/dl (1.69 mmol/l)
Low HDL cholesterol	
Men	< 40 mg/dl (1.04 mmol/l)
Women	< 50 mg/dl (1.29 mmol/l)
High blood pressure	≥ 130/85 mmHg
High fasting glucose	≥ 110 mg/dl (6.1 mmol/l)

The increasing prevalence of the metabolic syndrome and related disorders in the industrialized populations is an enormous challenge to preventive public health efforts (Joint National Committee 1997; Mokdad et al. 1999; Adult Treatment Panel III 2001; Ford et al. 2002). While medical treatment of these conditions is expensive and the results often unsatisfactory, increased exercise and certain changes in dietary habits would benefit almost everyone. Results of recent studies and public health policy statements emphasize abundant intake of fruits and vegetables, as well as fat-free and low-fat dairy products with increased intake of potassium, magnesium, calcium, fiber, and protein (WHO Study Group 1991; Joint National Committee 1997; Appel et al. 1997; Kotchen & McCarron 1998; Krauss et al. 2000). They also emphasize reduced intake of sodium salts, total fats, saturated fats, and cholesterol. Unfortunately, population-wide implementation of any

effective dietary intervention to combat the above-mentioned disorders has proven problematic.

Enrichment of salt or other widely used food items has been the method of choice for a population-wide supplementation of iodine, various vitamins, iron and other mineral nutrients. A marked increase in the levels of potassium and magnesium in a variety of food items has been produced by using potassium- and magnesium-enriched salt alternatives instead of common salt (Karppanen 1994; Karppanen & Mervaala 1996). The use of such foods produces lowering of elevated blood pressure and also other beneficial effects both in hypertensive animals and in man (Karppanen 1989; Mervaala et al. 1992; Geleijnse et al. 1994; Mervaala et al. 1994; Itoh & Kawasaki 1998; Katz et al. 1999).

Increased intake of plant sterols is another approach to dietary treatment of cardiovascular risk factors. The serum cholesterol-lowering effect of esterified plant sterols and stanols in patients with mild hypercholesterolemia is well established (Law 2000), and a whole new class of margarines and other fat-derived products containing these compounds has been accepted as part of a healthy antiatherogenic diet (Lichtenstein & Deckelbaum 2001). However, due to reactive increase of cholesterol synthesis in the liver, only a mild lowering of serum cholesterol by plant sterols is achieved, and, if the intake of fat is increased, also the risk of obesity will increase.

Recently, the protective effects of dietary calcium in osteoporosis and hypertension have been widely publicized (Bryant et al. 1999; McCarron & Reusser 1999); however, the possible cholesterol-lowering effects of calcium have created much less interest. Yet, an accumulating body of both experimental and clinical evidence since the 1950's indicates that calcium supplementation might be an effective, safe and economic means of reducing the serum levels of harmful lipids (Vitale et al. 1959; Yacowitz et al. 1965; Renaud et al. 1983; Denke et al. 1993), possibly even obesity (Zemel et al. 2000).

The purpose of the present study was to explore in the obese Zucker rat, a model of the metabolic syndrome, the possibilities of natural plant sterols and the mineral nutrients calcium, magnesium and potassium in the prevention and treatment of hyperlipidemia, hypertension and obesity, and study the mechanisms of the observed effects. A major focus of this study was on the effects of dietary calcium on serum lipids.

2 REVIEW OF THE LITERATURE

2.1 CALCIUM

Calcium is an essential nutrient, quantitatively the most abundant of the body's minerals and a vital electrolyte. Besides structural support, calcium is required for critical biological functions like nerve conduction, muscle contraction, cell adhesiveness, mitosis, and blood coagulation. Inadequate intake of calcium is a global problem, especially in aging populations, and it has been associated with several medical disorders, such as osteoporosis, hypertension, colon cancer, breast cancer, and kidney stones (for review, see Miller & Anderson 1999). Based on recent research, increased dietary intake of calcium is currently recommended for the general population to lower the risk of these chronic diseases (Krauss et al. 2000; Miller et al. 2001).

2.1.1 DIETARY INTAKE AND SOURCES OF CALCIUM

The Recommended Dietary Allowance (RDA) for calcium has long been 800 mg/d. Recognition of the many health benefits of calcium has led to increases in dietary calcium recommendations up to 1500 mg/d, depending on sex and age group (NIH Consensus Panel 1994; Bryant et al. 1999). Many population groups, however, consume even less than the RDA of calcium, especially in the USA (Fleming & Heimbach 1994). In Finland, the situation is clearly better; the mean intake of calcium is 913 mg/d in women and 1159 mg/d in men (National Public Health Institute 1998), but particularly among adolescent and older females calcium intake may still be inadequate.

Governmental and health professional organizations, as well as leading nutrition and medical experts, recommend food as the preferred source of calcium (NIH Consensus Panel 1994; Miller et al. 2001). By far the most important of dietary sources of calcium is milk. Milk has a high calcium content of 1200–1300 mg/l, and milk and other dairy products provide approximately 75% of the total calcium available in the food supply (National Public Health Institute 1998). Fruits, vegetables and cereals also contain calcium but to a much lesser extent. Hence, people who avoid milk products for some reason, such as lactose intolerance, may need to consider consuming calcium-fortified food items or calcium supplements (Miller et al. 2001).

In addition to the content of calcium in foods, the bioavailability of calcium from foods must be considered (Gueguen & Pointillart 2000). It ranges from a low of 5% in spinach to about 30% in milk and

more than 60% in broccoli (Weaver et al. 1999). However, high absorbability cannot overcome a low content: almost five kilos of broccoli needs to be consumed to get the same amount of calcium absorbed as from one liter of milk. The problem with most vegetables and cereals is that they contain oxalates and phytates, which inhibit the absorption of calcium (Weaver et al. 1999). On the other hand, industrially processed milk products often contain excess amounts of phosphates and sodium salts (National Public Health Institute 1998). Phosphate binds calcium in the intestine, and sodium linearly increases the excretion of calcium in urine (Nordin et al. 1993), even independently of calcium intake (Matkovic et al. 1995). Urinary calcium losses account for 50% of the variability of calcium retention (NIH Consensus Panel 1994). Therefore, foods with reduced sodium and increased calcium contents would appear most advantageous.

Calcium can also be obtained from commercial supplements. Calcium in supplements is found in various forms including calcium carbonate, citrate, citrate malate, gluconate, and lactate. In general, the absorption of calcium from the different salts is similar, but carbonate, which contains the highest percentage of calcium, is usually the least expensive and thus most cost-effective (Heaney et al. 2001). Some supplements may also contain vitamin D that helps in calcium absorption, and magnesium that as well is a vital electrolyte and may become poorly absorbed during high calcium intake.

However, what is absorbed is not the only important part of the ingested calcium. The non-absorbed fraction can bind many potentially harmful food ingredients, such as oxalic acid and saturated fatty acids, and the precipitation of bile acids with calcium may even contribute to lowering of the risk of atherosclerosis and colorectal cancer (see 2.1.3).

2.1.2 PHYSIOLOGY OF CALCIUM

An average adult body contains about 1,5 kg of calcium, 99% of it in the skeleton (for review, see Marx 1996). The calcium in the bone is in equilibrium with the calcium in the extracellular fluid, but only about 0.5% of the total pool is exchangeable. Calcium in blood is divided among protein-bound, complexed, and ionized or free fractions. The ionized fraction is the focus of metabolic control, especially through parathyroid hormone (PTH) and vitamin D, and it is kept constant at 1.0–1.2 mmol/l by processes that continuously add and remove calcium. Calcium enters the plasma via absorption from the intestine and resorption of from the bone mineral. Bone resorption and formation are tightly coupled, with approximately 500 mg of calcium entering and leaving the skeleton daily. Calcium leaves the extracellular fluid via secretion into the gastrointestinal tract, excretion in urine, deposition in bone mineral, and secretion in sweat.

Generally, less than half of dietary calcium is absorbed in adults (for review, see Gueguen & Pointillart 2000). Calcium absorption increases during growth, pregnancy, and lactation, and decreases with advancing age. Most of the calcium is absorbed in the proximal small intestine, and the efficiency of absorption decreases in the more distal intestinal segments. Active transport is more important in the upper intestine, and diffusion-limited absorption in the lower intestine. Both processes are influenced by vitamin D.

Secretion of calcium into the intestinal lumen occurs at a constant rate of 100–200 mg/d and is independent of absorption. In all, about 90% of the daily intake of calcium is excreted in the feces.

The amount of calcium excreted in the urine, about 50–300 mg/d, is normally less than 2% of that filtered by the glomerulus. This is due to very efficient reabsorption, which takes place predominantly in the proximal tubule and in Henle's loop and to a small extent in the distal tubule (for review, see Rouse & Suki 1990). The excretion of other electrolytes affects the urinary excretion of calcium. For example, urinary calcium is usually proportional to urinary sodium, and sulfate also increases calcium excretion.

The primary regulator of calcium metabolism is PTH; it acts directly on bone, where it induces calcium resorption, and on the kidneys, where it stimulates calcium reabsorption and synthesis of 1,25-dihydroxycholecalciferol, the active form of vitamin D that stimulates calcium absorption from the gastrointestinal tract (for reviews, see Dawson-Hughes 1996 and Guyton & Hall 1996). Serum PTH levels are tightly regulated by a negative feedback loop. Calcium, acting through the calcium-sensing receptor, and vitamin D, acting through its nuclear receptor, inhibit PTH synthesis and release. The homeostatic action of PTH can preserve blood calcium concentration acutely at the cost of bone destruction. Immediate control of blood calcium is probably due to the effects of the hormone on bone and, to a lesser extent, on renal calcium clearance. Maintenance of steady-state calcium balance, on the other hand, probably results from the effects of vitamin D on calcium absorption. This is mediated principally by regulation of the formation of a calcium-binding protein in the intestinal epithelial cells over a period of several days.

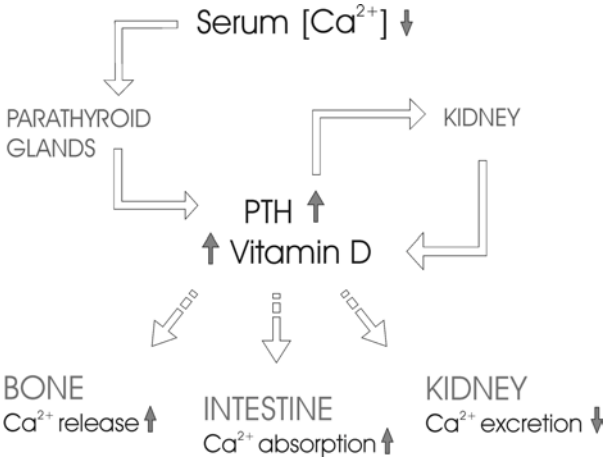


Fig 1. Regulation of calcium balance

Calcitonin is another hormone regulating calcium homeostasis (Guyton & Hall 1996). It is secreted by the thyroid gland, and in several mammalian species it acts as the physiologic antagonist to PTH; however, in humans the physiologic significance of calcitonin is questionable. The hypocalcemic activity of calcitonin is accounted for primarily by inhibition of osteoclast-mediated bone resorption and secondarily by stimulation of renal calcium clearance.

The physiology of calcium metabolism is also tightly coupled with phosphate absorption and excretion (Guyton & Hall 1996). Normally, when diet contains about equal amounts of calcium and phosphate, phosphate is partly excreted in the feces in combination with non-absorbed calcium, and the rest is readily absorbed into the blood and later excreted in the urine. In the kidney, about 80–90% of phosphate is reabsorbed, mainly in the proximal tubules. PTH, by augmenting

phosphate excretion via inhibition of this proximal reabsorptive process, plays a central role in phosphate homeostasis. When dietary phosphate intake increases, a transient rise in plasma phosphate concentration is usually observed. This results in a similarly transient reduction in the plasma ionized calcium level, due largely to deposition of calcium phosphate in bone, which stimulates PTH secretion. By enhancing fractional phosphate excretion, PTH restores external phosphate balance and normophosphatemia. Vitamin D probably opposes the phosphaturic actions of PTH in the renal tubules by augmenting phosphate reabsorption. Hypophosphatemia, on the other hand, decreases the rate of calcium uptake into bone, increases intestinal calcium absorption, and directly and indirectly stimulates bone breakdown.

2.1.3 CALCIUM AND SERUM LIPIDS

Considering the ever-growing amount of calcium-related research, particularly around hypertension and osteoporosis, it is surprising how little attention has been called upon the possible lipid-lowering effects of an increased intake of calcium.

Epidemiological evidence in favor of the hypothesis of calcium as a lipid-lowering agent is undeniably scarce. Higher calcium intake rather seems to correlate with higher serum cholesterol levels and incidence of coronary heart disease (Segall 1977; Sharlin et al. 1992; Artaud-Wild et al. 1993). This might be due to the fact that dietary calcium usually comes from milk products that often contain lots of saturated fat and cholesterol, which may override the effects of calcium. Conversely, low-fat and low-cholesterol diets, which lower serum lipid levels, often contain less calcium (Schectman et al. 1990; Holbrook & Barrett-Connor 1991). One fairly large cross-sectional study in 5 394 men and 4 800 women found a linear increase of both total cholesterol and HDL cholesterol with serum calcium levels, independent of confounding factors such as age, blood pressure, body weight, fat and cholesterol intake (De Bacquer et al. 1994). Recently, no association between milk consumption and coronary or all cause mortality was found in a 25-year prospective study on a cohort of 5 765 men in Scotland (Ness et al. 2001). In a large cohort study of 34 486 postmenopausal women, higher intake of calcium, but not of vitamin D or milk products, was associated with reduced ischemic heart disease mortality (Bostick et al. 1999). On the other hand, several studies indicate that milk and milk products have hypolipidemic and anti-atherogenic effects (Howard & Marks 1977; Pfeuffer & Schrezenmeir 2000), which could be related to calcium, but may also be due to other bioactive substances in milk (Sipola 2002).

However, there are intervention studies both with animals (TABLE 2) and humans (TABLE 3) that have explored the effects of dietary calcium on serum lipids.

In rats supplemented with dietary fat and cholesterol (Vitale et al. 1959; Fleischman et al. 1967; Yacowitz et al. 1967; Fleischman et al. 1972), consistent decreases in serum total cholesterol and triglycerides with increased calcium intakes were observed. The level of serum total cholesterol in rats fed 1.2% dietary calcium was about 30–40% lower and with 2.0% calcium up to 60% lower than in the controls fed low-calcium diets. Calcium was hypolipidemic during both saturated and polyunsaturated fat supplementation, but the effects were more

Table 2. Effects of dietary calcium on serum lipids in animals

Species	Diet; treatments; duration	Results	Reference
Rat	20% fat, 1% cholesterol, 0.3% cholic acid; [Ca] 0.2, 0.6 or 1.2%, [Mg] 0.02 or 0.2%; 4 weeks	TC ↓ PL ↓	Vitale et al. 1959
Rat	20% fat, 2% cholesterol; [Ca] 0.08, 0.2, 1.2 or 2%; 5 months	TC ↓ TG ↓ Fecal fat, bile acids and sterols ↑	Fleischman et al. 1967
Rat	20% fat, 2% cholesterol; [Ca] 0.08, 0.2 or 1.2%; 3 weeks	TC ↓ TG ↓ Fecal fat, bile acids and sterols ↑	Yacowitz et al. 1967
Rat	20% fat, 2% cholesterol; [Ca] 2.0 % ± vitamin D2; 3 weeks	TC ↓ TG ↓ Fecal fat and sterols ↑	Fleischman et al. 1972
Rabbit	Low-fat; [Ca] 0.02, 0.8 or 1.6%; 3 months	TC ↓ TG ↔	Iacono 1974
Rabbit	25% butter, 25% casein; [Ca] 0.3 or 0.9%, [Mg] 0.04 or 0.4%; 6 months	TC ↓ HDL-C ↔ TG ↓ Atherosclerosis ↓	Renaud et al. 1983
Rabbit	10% fat, 21% casein; [Ca] 0.8 or 1.4%; 7 weeks	TC ↓ PL ↔ Fecal fat ↑	Van der Meer et al. 1985
Rabbit	11% fat, 20% casein, cod, or soy protein; [Ca] 0.7 or 1.4%; 4 weeks	TC ↓ LDL-C ↓ HDL-C ↔ VLDL-C ↓ TG ↔ Fecal fat↑	Jacques et al. 1995
Pig	19% fat, 0.05% cholesterol; [Ca] 0.7 or 2.1% ± vitamin D; 6 weeks	No changes in TC, VLDL-C, LDL-C, HDL-C, or TG	Foley et al. 1990
Pig	10% butter, 0.5% cholesterol; [Ca] 0.7 or 1.4%; 15 days	TC ↓ LDL-C ↓ HDL-C ↔ Serum bile acids ↓	De Rodas et al. 1996
Goat	Fat and cholesterol supplemented goat milk; [Ca] 0.1% or 0.2%; 20 weeks	No changes in TC, VLDL-C, LDL-C, HDL-C, or TG	Diersen-Schade et al.1984
Goat	Normal goat milk; [Ca] 0.1% or 0.2% ± vitamin D3; 20 weeks	TC ↓ Atherogenesis ↓ Fecal fat ↔	Hines et al. 1985

[Ca] = total calcium content; TC = total cholesterol; VLDL-C, LDL-C, HDL-C = very low, low, high density lipoprotein cholesterol; TG = triglycerides; PL = phospholipids

pronounced in the presence of saturated fat (Yacowitz et al. 1967). Cholic acid enhanced the diet-induced hyperlipidemia and even abolished the effects of calcium (Vitale et al. 1959), but vitamin D had no significant effect on either direction (Fleischman et al. 1972). In New Zealand white rabbits fed a low-fat diet, calcium supplementation lowered serum total cholesterol and reduced the accumulation of cholesterol in heart, kidney and muscles, but the tissue triglyceride levels were increased (Iacono 1974). In a six-month experiment with butterfat-supplemented rabbits, Renaud et al. (1983) found that calcium and magnesium, in addition to lowering of cholesterol and triglycerides, also reduced platelet aggregation, the severity of atherosclerosis and accumulation of cholesterol in the aorta. Casein and cod protein -induced hypercholesterolemia in rabbits was effectively counteracted by 1.4% dietary calcium, while soy protein did not raise serum lipid levels at all (Van der Meer et al. 1985; Jacques et al. 1995). In fat- and cholesterol-supplemented pigs the effects of calcium were less unanimous. Tripling the dietary calcium intake had no effect on cholesterol or triglyceride concentrations in plasma or their partitioning among plasma lipoproteins (Foley et al. 1990). The levels of both dietary and plasma cholesterol in this study, though, were only a fraction of those in the other pig study (De Rodas et al. 1996), where the lowering of serum total and LDL cholesterol after dietary cholesterol supplementation was enhanced by a diet containing 1.4% calcium. Another species where findings seem to differ is young goat. Diersen-Schade et al. (1984) found no effect of dietary fat or calcium on plasma and lipoprotein cholesterol concentrations, but a year later Hines et al. (1985) reported calcium-induced lowering of cholesterol in plasma and tissues in a similar setting. High intake of vitamin D, however, reversed the beneficial effects of calcium, resulting in a marked increase in lipid accumulation in the aorta.

The first clinical studies in the 1960's and 1970's were rather small but well conducted and showed a consistent serum cholesterol- and triglyceride-lowering effect of increased dietary calcium intake. In healthy adults on normal diets, a moderate 890 mg/d calcium supplementation produced a mean 15.4 mg/dl (0.4 mmol/l) decrease in serum total cholesterol and 32.2 mg/dl (0.83 mmol/l) decrease in triglycerides (Yacowitz et al. 1965). The decreases were greater, up to 15–30% in those whose baseline lipid levels were elevated. Calcium counteracted the rise of serum cholesterol induced by saturated fat; polyunsaturated fat supplementation did not raise the cholesterol levels (Bhattacharyya et al. 1969). In hyperlipidemic patients, Carlson et al. (1971) found a 10% decrease in serum cholesterol during calcium treatment, but no change in triglycerides; Bierenbaum et al. (1972) reported a calcium-induced decrease in all serum lipids over 12 months; and Lehtonen & Viikari (1979) showed that calcium supplementation was able to enhance the lipid-lowering effect of clofibrate. In children with familial hypercholesterolemia, calcium supplementation in addition to a low-fat and low-cholesterol diet produced a rather small but significant 4% decrease in serum LDL cholesterol and a 4% increase in serum apolipoprotein A1, which is the main protein in HDL (Groot et al. 1980). Similar results were achieved in adults with only 400 mg/d increase in calcium intake, in addition to the low-fat, low-cholesterol diet (Bell et al. 1992). Bierenbaum et al.

Table 3. Effects of dietary calcium on serum lipids in humans

Subjects	Design, duration	Intervention	Result	Reference
13 healthy adults	Cross-over, 3 weeks	Ca 890 mg/d	TC ↓ TG ↓ Fecal fat ↑	Yacowitz et al. 1965
12 healthy men	Cross-over, 4 x 2 weeks	SAFA/PUFA 90g+Ca 2 g/d	TC ↓ Fecal fat and C ↑	Bhattacharyya et al. 1969
16 HC patients	Cross-over with placebo, single-blind, 4+8 weeks	Ca 2 g/d	TC ↓ TG ↔	Carlson et al. 1971
20 HC patients	Randomized, open, 12 months	Ca 800 mg/d	TC ↓ TG ↓	Bierenbaum et al. 1972
30 HC patients	Cross-over with placebo, single-blind, 16 months	Clofibrate + Ca 800 mg/d	VLDL-C ↓ LDL-C ↓ HDL-C ↔ TG ↔	Lehtonen & Viikari 1979
50 FHC children	Cross-over with placebo, double-blind, 10+10 weeks	Ca 400 mg/d	LDL-C ↓ Apo-AI ↑	Groot et al. 1980
43 hypertensive 27 normotensive	Randomized, double-blind, cross-over, 8+8 weeks	Ca 1 g/d	TC, VLDL-C, LDL-C, HDL-C, and TG: ↔	Karanja et al. 1987
200 healthy adults	Open, 6 months	Ca 1,4 g/d	TC ↓ HDL ↔ TG ↔	Bierenbaum et al. 1987
56 HC patients	Randomized, double-blind, cross-over, 6+6 weeks	AHA Step-1 + Ca 1,2 g/d	LDL-C ↓ HDL-C ↑	Bell et al. 1992
13 healthy men	Randomized, single-blind, cross-over, 10+10 days	Ca 1,8 g/d	TC ↓ LDL-C ↓ Apo-B ↓ TG ↔ HDL-C ↔ Apo-A1 ↔ Fecal fat↑ bile acids ↔	Denke et al. 1993
130 hypertensive 196 normotensive	Randomized, parallel, placebo-controlled, 12 weeks	Ca 1 g/d	TC, VLDL-C, LDL-C, HDL-C, and TG: ↔	Karanja et al. 1994
10 healthy men	Randomized, double-blind, cross-over, 2+2 weeks	Ca 900 mg/d	LDL-C ↓ HDL-C ↔ TG ↔ Fecal fat and bile acids ↑	Shahkhalili et al. 2001
223 elderly women	Randomized, placebo-contr, 1 year	Ca 1 g/d	TC ↔ LDL-C ↓ HDL-C ↑ TG ↔	Reid et al. 2002

(F)HC = (familial) hypercholesterolemia; Ca = supplemental elementary calcium; SAFA/PUFA = saturated/polyunsaturated fatty acids; C = cholesterol; TC = total cholesterol; LDL-C, VLDL-C, HDL-C = low, very low, high density lipoprotein cholesterol; Apo = apolipoprotein; TG = triglycerides

(1987) had 200 volunteers drink one liter of calcium-fortified milk per day for six months and reported, in addition to 7/6 mmHg decrease in blood pressure, a slight decrease in serum total cholesterol. Two smaller studies have examined the effects of dietary calcium fortification on serum and fecal lipid contents. Increased intake by 1800 mg/d resulted in decreases of 6% in total and 11% in LDL cholesterol and doubled the saturated fat excretion (Denke *et* al. 1993), and supplementation of 900 mg/d of calcium in chocolate markedly reduced the absorption of cocoa butter, and lowered plasma LDL cholesterol by as much as 15% (Shahkhalili *et* al. 2001). Very recently, a 1 g/d calcium supplementation was reported to produce a 7% increase in HDL cholesterol and 6% decrease in LDL cholesterol in a study on 223 healthy postmenopausal women – the group most commonly using calcium supplements (Reid *et* al. 2002). However, not all studies have demonstrated any hypolipidemic effects for calcium. Two fairly large intervention studies by the same group (Karanja *et* al. 1987; Karanja *et* al. 1994) found that hypertensive patients at baseline consumed less calcium, magnesium and potassium than normotensive patients and had significantly lower HDL cholesterol and higher LDL cholesterol levels, but no changes occurred in plasma lipids or lipoproteins with either calcium supplementation or counseling to increase dietary calcium intake.

Mechanisms of the effects

The possible hypolipidemic mechanisms of calcium include **1)** inhibition of the intestinal absorption of cholesterol (Fleischman *et* al. 1967; Yacowitz *et* al. 1967; Bhattacharyya *et* al. 1969; Fleischman *et* al. 1972; Diersen-Schade *et* al. 1984), **2)** inhibition of absorption of bile acids (Fleischman *et* al. 1967; Yacowitz *et* al. 1967; De Rodas *et* al. 1996; Shahkhalili *et* al. 2001), and **3)** inhibition of absorption of fat (Yacowitz *et* al. 1965; Fleischman *et* al. 1967; Yacowitz *et* al. 1967; Bhattacharyya *et* al. 1969; Fleischman *et* al. 1972; Van der Meer *et* al. 1985; Denke *et* al. 1993; Jacques *et* al. 1995; Shahkhalili *et* al. 2001).

Binding bile acids is a mechanism familiar from the effect of cholestyramine and other resins (Witztum 1996). The catabolism of cholesterol to bile acids is an important route for the elimination of cholesterol from the body, accounting for approximately 50% of the cholesterol eliminated daily (St-Pierre *et* al. 2001). Increased fecal loss of bile acids results firstly in decreased absorption of fat and cholesterol and secondly in increased conversion of cholesterol to bile acids in the liver. Even if *de novo* synthesis of cholesterol is increased, it may not be sufficient for the replacement bile acid synthesis, and cholesterol must be taken from the circulation via LDL-receptors, which leads to lowering of serum LDL cholesterol.

Increased fecal loss of fat, especially saturated fat, is also important because saturated fatty acids, when absorbed, will inhibit the receptor-mediated uptake of LDL into liver cells, thereby decreasing the clearance of LDL particles from the circulation (Kris-Etherton *et* al. 1988; Grundy & Denke 1990). Consequently, decreased absorption of saturated fat would lead to a decrease in serum cholesterol. Calcium and other divalent cations may bring about this effect through precipitation of saturated fatty acids from the solution. This concept of formation of non-absorbable calcium and magnesium soaps in the intestine was first proposed by Givens (1917), and has since been

confirmed in both animal and human studies (Drenick 1961; Welberg et al. 1994). The bile and fatty acid binding properties of calcium are also utilized in the prevention of colorectal neoplasia (Baron et al. 1999; Holt 1999).

In summary, despite some controversy and lack of large epidemiological and intervention studies, the existing evidence strongly suggests that calcium does have beneficial effects on serum lipids, in animals as well as in humans. Larger-scale prospective clinical trials need to be conducted to define the scope and significance of this effect.

2.1.4 CALCIUM AND BLOOD PRESSURE

After 20 years of intense investigation in the area of dietary calcium and blood pressure, a consensus is at hand: a large body of recent data consistently and clearly prove the antihypertensive effect of increased intake of calcium (for review, see McCarron & Reusser 1999). The association between higher dietary calcium intake and lower prevalence of high blood pressure was first reported by McCarron et al. (1984) in the analysis of the first National Health and Nutrition Examination Survey (NHANES I). Since then, more than 30 well-designed epidemiological studies assessing the calcium-blood pressure relationship have been published. A meta-analysis of 23 observational studies estimated that each 100 mg increase in daily calcium intake would produce a lowering of 0.39 mmHg in systolic and 0.35 mmHg in diastolic blood pressure (Birkett 1998).

Studies in various strains of hypertensive rats and their normotensive counterparts have largely confirmed the initial hypothesis that an increase in dietary calcium intake reduces blood pressure, and provided some insight into its possible mechanisms of action (for review, see Hatton & McCarron 1994; Schleiffer & Gairard 1995). Calcium may act concurrently through several physiological mechanisms, including reduced membrane permeability and intracellular calcium, changes in calcium-regulating hormones, modulation of the sympathetic nervous system, and altering the metabolism of other electrolytes (Hatton et al. 1995). Pörsti (1991) found that the urinary excretion of sodium increased and the action of deoxycorticosterone on sodium balance was prevented (Pörsti et al. 1991), and that the activity of erythrocyte cell membrane calcium-ATPase was increased, platelet intracellular free calcium reduced, vascular smooth muscle relaxation improved and contractile responses attenuated by a high-calcium diet in spontaneously hypertensive rats (Pörsti et al. 1990; Pörsti 1992). These results were later confirmed, and complemented by new observations indicating that the augmented endothelium-dependent vasorelaxation could be explained by enhanced hyperpolarization, mediated via opening of calcium-activated potassium channels, increased sensitivity to nitric oxide in arterial smooth muscle, and decreased production of superoxide and vasoconstrictor prostanoids (Mäkynen et al. 1996; Tolvanen et al. 1998; Jolma et al. 2000).

The antihypertensive effect of increased dietary calcium intake may also be mediated via suppression of the calcitrophic hormones PTH and vitamin D (Zemel 2001). These hormones increase vascular smooth muscle intracellular calcium, which leads to increased peripheral resistance and blood pressure

(FIGURE 2). Dysregulation of calcium homeostasis may also be a fundamental factor linking together hypertension and obesity (Zemel 2001).

More than 60 calcium intervention trials in humans have been reported, and as with all nutrient modification trials, the results have been heterogeneous (Luft & McCarron 1991). However, a most carefully conducted and recent meta-analysis of 43 randomized controlled studies found that both dietary and supplemental increase in calcium intake to more than 1000 mg/d for at least two weeks leads to a significant reduction in blood pressure (Griffith et al. 1999). Pooled estimates across all studies, comprising 3500 subjects altogether, showed decreases of 1.44 mmHg in systolic and 0.84 mmHg in diastolic blood pressure. The decreases tended to be greater and less heterogeneous in dietary calcium studies as compared with those that employed non-food sources of calcium (Griffith et al. 1999). Perhaps the most important recent study in this area is the Dietary Approaches to Stop Hypertension (DASH) trial (Appel et al. 1997). The DASH diet, rich in calcium, magnesium, potassium and fiber, produced up to 11.4/5.5 mmHg reductions in blood pressure and, when analyzed statistically for calcium effect, the observed reductions in blood pressure correspond remarkably with those revealed by meta-analyses of both epidemiological and clinical intervention trials (McCarron & Reusser 1999). On the whole, these data support the role of the combination of the mineral nutrients as an independent factor that significantly contributes to the lowering of hypertension risk.

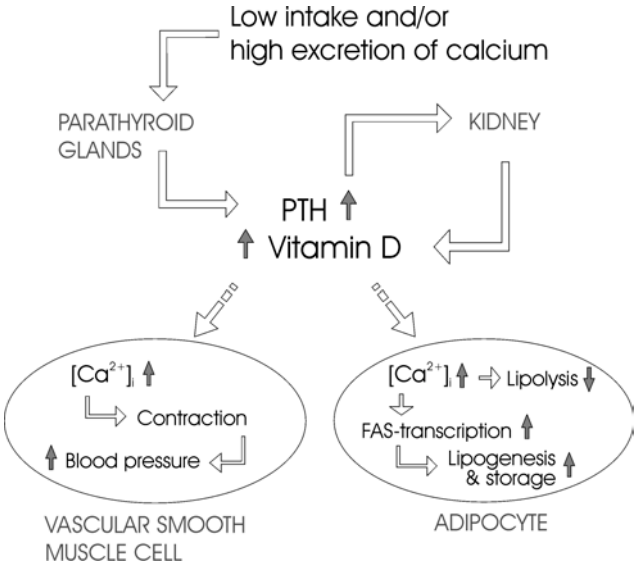


Fig 2. Suggested mechanism of the effects of dietary calcium on blood pressure and obesity (Zemel 2001)

2.1.5 CALCIUM AND OBESITY

Obesity is the common denominator in several comorbid conditions, including hypertension, hyperlipidemia and type II diabetes, and its prevalence is increasing rapidly all over Western world (for review, see Anderson & Konz 2001). While it is known that weight reduction would be one of the most effective means of lowering the risk of cardiovascular diseases – even a few per cent decrease in body weight is associated with significant lowering of blood pressure and improvement of glucose tolerance and blood lipid profile (Anderson & Konz 2001) – the treatment of obesity in practice has proven extremely difficult.

Reductions in body weight gain were observed in several of the previously cited experimental calcium supplementation studies (Fleischman et al. 1967; Yacowitz et al. 1967; Foley et al. 1990; Pörsti et al. 1990; Mäkynen et al. 1996). There are two plausible explanations: dietary calcium

may form soaps with fatty acids and thereby prevent the absorption of some of the fatty acids released during lipid digestion, and/or dietary calcium may bind bile acids, which would decrease micelle formation and thus reduce lipid absorption and digestible energy of the diet. Many of these studies also reported increases in fecal fat excretion, which supports these assumptions.

Among the clinical studies addressing the effect of calcium on serum lipids, Carlson et al. (1971) reported a significant 0.55 kg weight loss by calcium supplementation of 2 g/d for eight weeks, and Karanja et al. (1994) found a small but significant decrease in body weight by 1 g/d calcium supplementation in both men and women in 12 weeks. In the same study, counseling to increase dietary calcium through food consumption to 1.5 g/d resulted in substantial increase in energy intake, yet body weight was not significantly altered. Neither did Shahkhalili et al. (2001) find any significant changes in body weight during two weeks' consumption of calcium-fortified chocolate. The other studies listed in TABLE 3 did not report body weights. In a clinical trial investigating the antihypertensive effect of calcium in obese African-Americans, an increase in calcium intake from about 400 to 1000 mg/d for one year resulted in as much as 4.9 kg reduction in body fat (Zemel et al. 2000). A recent re-evaluation of five clinical studies, designed with a primary skeletal end point on a total of 780 women in their 3rd, 5th and 8th decades, showed significant negative associations between calcium intake and body weight in all age groups across nearly four years of observation (Davies et al. 2000). Estimates of the relationship indicated that a 1000 mg daily calcium intake difference is associated with as much as 8 kg difference in body weight and that calcium intake explains approximately 3% of the variance in body weight.

Epidemiological analysis of both the NHANES I (McCarron et al. 1984) and NHANES III databases have revealed an inverse association with body weight and dietary calcium intake (Zemel et al. 2000). Moreover, calcium adjusted for energy intake had a negative relationship with two-year changes in total body weight and body fat in young women in a prospective analysis of an exercise intervention study (Lin et al. 2000). Also a recent longitudinal study in preschool children showed that higher intake of calcium was associated with lower body fat (Carruth & Skinner 2001).

Recently, a new theory about regulation of intracellular calcium as a fundamental factor linking together obesity, insulin resistance and hypertension has been presented (FIGURE 2; Zemel 1998; Zemel 2001). It was discovered that increasing intracellular calcium concentration stimulates the expression and activity of fatty acid synthase (FAS) and inhibits lipolysis in human and murine adipocytes via a calcium-dependent mechanism (Jones et al. 1996; Xue et al. 1998). Moreover, vitamin D and PTH were shown to produce sustained increases in adipocyte intracellular calcium and a corresponding inhibition of lipolysis (Zemel et al. 2000). This phenomenon may also contribute to the high body weight and fat mass reported in primary hyperparathyroidism (Grey et al. 1994). Increased intracellular calcium in fat and muscle cells may even interfere with signal transduction and attenuate insulin response (Zemel 1998). The levels of PTH and vitamin D are elevated during low intakes of calcium. An increase in dietary

calcium intake should, accordingly, suppress these calcitrophic hormones and thereby reduce intracellular calcium concentration and lipid storage in adipocytes; these effects together with reduction in body weight were indeed observed in calcium supplemented obese mice (Shi et al. 2001).

In conclusion, the possible role of calcium in the regulation of adiposity is a fairly novel idea and not usually quoted in calcium-related reviews. It has a plausible physiological basis and a good deal of experimental as well as some recent clinical and population data to support it. However, larger intervention studies should be performed to define the true significance of this action.

2.1.6 ADVERSE EFFECTS OF CALCIUM

Dietary calcium intake up to 2000 mg/d is generally recognized as safe (NIH Consensus Panel 1994). However, no research data in humans are available about the long-term risks or benefits of consuming higher amounts. Clinical toxicity of excessive calcium intakes could rise from development of hypercalcemia; symptoms may include confusion, fatigue, irritability, in severe cases even cardiac arrhythmias and, particularly with associated hyperphosphatemia, soft tissue calcification and renal damage (Spiegel 1996). True life-threatening toxicity is rare: there are about 30 case reports from the last two decades of the milk-alkali syndrome, and most of them had a predisposing factor, such as thiazide diuretic treatment or concurrent high alkali intake (Whiting & Wood 1997). High calcium intake has also been thought to cause kidney stones, for which hypercalciuria is an important risk factor (Whiting & Wood 1997). However, more recent studies suggest that increased dietary calcium may actually reduce the risk of nephrolithiasis (Heller 1999). Although dietary calcium increases urine calcium, it also binds phosphate and oxalic acid in the intestine, thereby reducing the formation of calcium oxalate and calcium phosphate crystals in the kidney. A normal rather than low calcium intake provided protection against recurrent stones even in patients with idiopathic hypercalciuria (Borghi et al. 2002). Moreover, with long-term consistent increase in calcium intake, intestinal adaptation mechanisms reduce the fractional calcium absorption, which already is highly dependent on the dietary source (Weaver et al. 1999). On the other hand, while reducing the likelihood of adverse effects, this adaptation may also reduce the expected benefits of calcium supplementation.

Another possible adverse effect of excessive calcium intake is the interaction with the absorption of other essential minerals, such as iron, magnesium, and zinc (Whiting & Wood 1997). The interaction between calcium and iron has been extensively studied. Calcium clearly inhibits iron absorption in a dose-dependent manner and, surprisingly, even heme iron absorption is inhibited (Hallberg et al. 1991). A recent review, however, concluded that while this is true in short-term absorption studies, long-term consumption of calcium supplements does not affect overall iron status (Bendich 2001). Calcium supplements may also affect the magnesium status of the body. It has been proposed that a dietary calcium to magnesium ratio greater than five may pose a risk for magnesium deficiency, partly because of reduced absorption but also because of increased excretion (Seelig 1994). This emphasizes the importance of

adequate or increased magnesium intake concurrently with calcium supplementation. In practice, though, high-calcium diets have not been demonstrated to affect magnesium retention in the long-term, probably due to the powerful compensatory function of the kidneys to decrease magnesium excretion (Whiting & Wood 1997). As for zinc, early animal studies indicated that high-calcium diets decrease its bioavailability, but most later studies in humans have not found any effect of calcium on either the absorption or whole-body retention of zinc (Whiting & Wood 1997).

Particularly calcium supplements can produce gastrointestinal side effects like bloating and constipation; they can also diminish the effectiveness of some medications such as alendronate, used to treat osteoporosis, or the antibiotic tetracycline, and should therefore not be consumed at the same time (Miller et al. 2001).

In conclusion, little evidence exists for any general toxicity of calcium intakes of even more than 2 g/d, especially if consumed with food when most of the calcium is not even absorbed. However, patients with hypercalcemia, thiazide treatment, or propensity to hypercalciuric stone formation should be more careful to keep to the recommended range.

2.2 MAGNESIUM

Magnesium is the most abundant intracellular divalent cation. It is an essential cofactor for a multitude of enzymatic reactions that are important for the generation of energy from ATP and for physiologic processes including neuromuscular function and maintenance of cardiovascular tone (for review, see Saris et al. 2000).

The serum concentration of magnesium is tightly regulated within a narrow range of 0.7–1.1 mmol/l as a result of the efficient absorption of dietary magnesium by the small intestine and conservation of magnesium in the kidney. The current RDA for magnesium is 400 mg/d; in Finland, the mean daily intake is about 400 mg in men and 300 mg in women (National Public Health Institute 1998). About 30% of dietary magnesium is absorbed in the small intestine, but this fraction can be substantially increased when intake is reduced. Approximately 96% of filtered magnesium is reabsorbed along the nephron, and only 4% is excreted into the urine. Hypercalcemia and hypercalciuria decrease tubular reabsorption of magnesium. Also excessive sodium intake and certain drugs, particularly thiazide diuretics, increase magnesium loss into the urine. There appears to be no specific and direct endocrine control of magnesium balance, similar to what exists for calcium, sodium and potassium. The calcitrophic hormones PTH, vitamin D and calcitonin, however, have similar actions for calcium and magnesium (Saris et al. 2000).

Magnesium deficiency may play an important role in the pathogenesis of several cardiovascular diseases, including cardiac arrhythmias, ischemic heart

disease, congestive heart failure, vascular complications of diabetes, hypertension and stroke (Arsenian 1993; Liao et al. 1998). Current dietary recommendations include the maintenance of adequate intake of magnesium, along with calcium and potassium, in order to lower the risk of cardiovascular diseases (Krauss et al. 2000).

2.2.1 MAGNESIUM AND LIPIDS

Several studies have evaluated the effects of dietary magnesium on serum and tissue lipids. In an early study in rats, 0.2% magnesium supplementation had no effect on serum cholesterol, but it effectively reduced tissue calcification and vascular lipid accumulation (Vitale et al. 1959). In a six-month experiment with cholesterol-fed rabbits (Renaud et al. 1983), 0.4% dietary magnesium markedly lowered serum total cholesterol and reduced the accumulation of cholesterol as well as the severity of atherosclerosis in the aorta. Another study in rabbits (Ouchi et al. 1990) showed no changes in cholesterol levels but, again, additional magnesium dose-dependently decreased both the area of aortic lesions and the cholesterol content in the aortas. Altura et al. (1990) demonstrated up to 40% lowering of cholesterol and triglyceride levels along with marked attenuation of the atherosclerotic process by magnesium supplementation in cholesterol-fed rabbits. Recently, increased magnesium intake was reported to reduce both cholesterol and triglyceride levels and inhibit atherogenesis in apo-E deficient mice receiving a low-fat diet (Ravn et al. 2001).

In humans, two similar randomized, single-blinded, controlled studies with 430 (Singh et al. 1990) and 400 patients (Singh et al. 1991) showed about 10% decreases in serum total and LDL cholesterol and triglyceride concentrations as a result of an increase in dietary magnesium intake from about 400 to 1000 mg/d. HDL cholesterol remained mostly unchanged, however, in originally hypomagnesemic subjects the dietary change induced an 11% increase in serum HDL cholesterol together with the decrease in the other lipids (Singh et al. 1990). A 500 mg/d oral magnesium supplementation lowered serum triglycerides but had no positive effect on cholesterols in a study with 69 hyperlipidemic patients on a low-fat, low-cholesterol diet (Kisters et al. 1993). Recently, magnesium supplementation was found to reduce both serum total and LDL cholesterol levels and insulin-stimulated glucose uptake in patients with type I diabetes (Djurhuus et al. 2001).

Thus, in the light of current knowledge, increased intake of dietary magnesium is likely to have beneficial effects on serum lipids. Moreover, experimental data suggest that it may attenuate the development of atherosclerosis even without major changes in the lipoprotein levels, especially in magnesium-deficient subjects. Magnesium deficiency is not uncommon, although it is often clinically latent, because less than 1% of total body magnesium is present in blood, and assessment of tissue magnesium status is problematic (Elin 1994). The mechanisms of the lipid-lowering effect of magnesium are poorly understood. In theory, as a divalent cation similar to calcium, it could bind fatty acids and bile acids in the intestine, reduce the absorption of saturated fat, and increase the excretion of cholesterol as bile acids from the liver. One of the above-mentioned studies (Renaud et al. 1983) reported a magnesium-induced increase in fecal fat excretion, but currently no data

are available on concomitant effects on bile acid excretion and serum lipids or atherogenesis.

2.2.2 MAGNESIUM AND BLOOD PRESSURE

Magnesium may also have a beneficial effect on blood pressure. No quantitative analyses are available, but qualitative overviews on epidemiological studies point to an inverse relationship between dietary magnesium intake and blood pressure (Whelton & Klag 1989; Mizushima et al. 1998). The large cross-sectional Atherosclerosis Risk in Communities (ARIC) study on 15 000 middle-aged Americans showed a negative correlation of dietary and serum magnesium levels to both systolic and diastolic blood pressure (Ma et al. 1995); on the other hand, no such relationship was found in the NHANES III (Hajjar et al. 2001). Data from clinical studies are also inconsistent. Dietary magnesium supplementation has been shown to lower blood pressure in many (Dyckner & Wester 1983; Widman et al. 1993; Kawano et al. 1998) but not in all (Cappuccio et al. 1985; Ferrara et al. 1992; Sacks et al. 1998) intervention studies.

2.2.3 MAGNESIUM AND OBESITY

Little is known about the effects of dietary magnesium on obesity, and no reductions of body weight attributable to increased magnesium intake were reported in any of the studies referred to above. Theoretically, magnesium could have an anti-obesity effect similar to calcium, because it too can form soaps with fatty acids in the intestine and thus reduce the digestible energy content of the diet (Givens 1917; Drenick 1961).

Furthermore, there is both experimental (Balon et al. 1995) and epidemiological evidence (Kao et al. 1999) that magnesium may attenuate the development of insulin resistance and type II diabetes, and insulin response has been reported to be improved by magnesium administration (Paolisso et al. 1989); this should also eventually affect adiposity. Interestingly, in a recent study obese persons were found more insulin-resistant and had lower magnesium concentrations in their serum and erythrocytes than nonobese controls (Zemva & Zemva 2000). Also the ARIC study reported an inverse relation between serum magnesium concentration and body mass index at baseline (Kao et al. 1999).

2.2.4 ADVERSE EFFECTS OF MAGNESIUM

The therapeutic window of magnesium is wide and severe toxic effects are extremely rare (for review, see Saris et al. 2000). Oral magnesium supplementation has a laxative effect that is even utilized in treatment of constipation, but large doses may also cause diarrhea and abdominal cramps. Signs of magnesium toxicity are vomiting, hypotension, bradycardia and other arrhythmias, somnolence, and weakness; these usually occur at plasma levels of four to five times higher than normal, and have only been observed during intravenous magnesium treatment. Magnesium toxicity is increased in patients with hypocalcemia, hyperkalemia and renal failure. Calcium gluconate is clinically used as an antidote for magnesium; therefore, with concurrent dietary supplementation of calcium and magnesium, any adverse effects attributable to magnesium are unlikely.

2.3 POTASSIUM

Potassium is the principal intracellular cation and mainly involved in membrane potential and electrical excitation of nerve and muscle cells. The extracellular concentration of potassium is kept constant at 4–5 mmol/l by active ion transport systems, which are usually coupled with the regulation of sodium concentration and excretion as well. Until recently, from an evolutionary point of view, humans consumed a diet low in sodium (about 0.5–1 g/d) and high in potassium (8–10 g/d), but these days the relation has often turned the other way around, largely as a result of the increasing consumption of industrially processed foods (Eaton & Konner 1985; Tobian 1997). The current Finnish recommendation for potassium intake is 3.5 g/d, and mean daily intake about 4.2 g in men and 3.3 g in women, whereas the intake of sodium is 3.7 g and 2.5 g, respectively (National Public Health Institute 1998).

In addition to its widely accepted role in the prevention and treatment of hypertension, high intake of potassium may also have other beneficial effects that are independent of blood pressure – for example, reduction of the risk of stroke, prevention of renal vascular, glomerular and tubular damage, and improvement in glucose intolerance (for review, see He & MacGregor 2001). In the Scottish Heart Health Study, findings included an unexpectedly powerful protective relation of dietary potassium to all cause mortality (Tunstall-Pedoe et al. 1997). Potassium also decreases urinary calcium excretion and thereby has many effects comparable to increased calcium intake, such as reduced risk of osteoporosis and kidney stones. Increasing potassium intake and reducing sodium intake have an additive effect in most of these conditions (He & MacGregor 2001).

2.3.1 POTASSIUM AND LIPIDS

There appears to be no direct evidence of any effect of dietary potassium on serum lipid levels in humans or in animals. However, potassium may protect against atherosclerosis by modifying lipid properties even without significant changes in their concentrations. In cholesterol-fed rabbits, increasing dietary potassium from 0.4% to 1.5% did not produce any differences in plasma cholesterol or body weight in six weeks, yet substantially reduced atherosclerotic lesions in coronary arteries (Ma et al. 1999). Other experimental studies have provided explanations for possible mechanisms by which increased intake of potassium may protect against cardiovascular diseases. Potassium has been shown to 1) inhibit free radical formation from vascular endothelial cells and macrophages (McCabe et al. 1994), which could also affect LDL oxidation and thereby the development of atherosclerosis; 2) inhibit proliferation of vascular smooth muscle cells (McCabe & Young 1994); and 3) inhibit platelet aggregation and arterial thrombosis (Lin & Young 1994). Recently, a high level of dietary potassium was shown to inhibit neointimal proliferation after balloon angioplasty in the rat carotid artery (Ma et al. 2000) as well as in a swine coronary artery (Ma et al. 2001).

2.3.2 POTASSIUM AND BLOOD PRESSURE

The important role of potassium intake in regulating blood pressure in both the general population and people with high blood pressure has been repeatedly shown

in epidemiological as well as clinical studies, and is today well established (Kotchen & McCarron 1998; He & MacGregor 1999). For instance, pooled data from a meta-analysis of 33 trials (2609 patients) on the effects of potassium supplementation showed highly significant net decreases of 4.5 mmHg in systolic and 2.5 mmHg in diastolic blood pressure with a median dose of 1.9 g/d (Whelton et al. 1997). Very recently, an analysis of the NHANES III database on 17030 subjects again confirmed that higher potassium intake is associated lower systolic and diastolic blood pressure (Hajjar et al. 2001).

The blood pressure-lowering effect has been particularly prominent in patients and animals with salt-sensitive hypertension (Karppanen 1991; Tobian 1997); thus, the action of potassium could be related to facilitation of natriuresis and decrease in volume load. However, the antihypertensive effect is most likely multifactorial; suggested mechanisms include reduced sympathetic nervous activity (Fujita & Sato 1992) and decreased pressor response to noradrenaline and angiotensin II (Campbell & Schmitz 1978). Experimental studies with a potassium-enriched mineral salt have supported the volume load theory (Mervaala et al. 1992), but also indicated that potassium may attenuate the vascular contractile responses and improve both endothelium-dependent and -independent relaxation (Mervaala et al. 1994). Recently, Tolvanen et al. (1998) showed that dietary potassium supplementation improved endothelium-dependent arterial relaxation in spontaneously hypertensive rats by mechanisms involving enhanced hyperpolarization, increased smooth muscle sensitivity to nitric oxide and decreased production of vasoconstrictor prostanoids. Increased intake of calcium together with potassium was more effective than either one alone in reducing blood pressure and restoring arterial tone. In another recent study, the most effective protection against hypertension induced by cyclosporin toxicity was achieved when dietary potassium and magnesium supplementations were combined (Pere et al. 2000).

2.3.3 POTASSIUM AND OBESITY

There are no reports in the literature that would closely link together dietary potassium and obesity, and only sparse references to point to even a distant connection. However, as with magnesium, type II diabetes might be the common link: improvements in carbohydrate metabolism in response to potassium administration have been reported (Rapoport & Hurd 1964; McFarland & Carr 1977; Karppanen et al. 1984). Furthermore, decreased skeletal muscle potassium and increased sodium to potassium ratios have been measured in obese and glucose intolerant men (Landin et al. 1988; Landin et al. 1991). A recent study demonstrated that insulin and leptin, hormones that reduce food intake and body weight in lean but not obese Zucker rats, hyperpolarize hypothalamic glucose-responsive neurons by opening ATP-sensitive potassium channels, suggesting that this potassium channel is involved in the physiological regulation of energy homeostasis (Spanswick et al. 2000). There is no evidence to date about any effect of dietary potassium on the function of this channel, but knowing that potassium supplementation can enhance hyperpolarization in vascular smooth muscle cells via its action on potassium channels (Tolvanen et al. 1998), this kind of a mechanism could be speculated.

2.3.4 ADVERSE EFFECTS OF POTASSIUM

The renal mechanisms for potassium excretion adapt efficiently to increases in the rate of potassium influx to extracellular fluid, particularly from dietary sources. Hence acute or chronic hyperkalemia due to exogenous potassium intake is uncommon, but usually occurs as a result of either potassium release from cells or decreased renal excretion (for review, see Kokko 1996). However, iatrogenic hyperkalemia may result from excessive parenteral potassium replacement, in patients with renal insufficiency, or with certain drugs like potassium-sparing diuretics and ACE-inhibitors. Particularly potassium supplements may be hazardous, and case reports of even severe hyperkalemia following abundant ingestion of potassium chloride either as salt substitutes or tablets have been published (Schim van der Loeff HJ et al. 1988; McCaughan 1984; Browning & Channer 1981). Moreover, potassium chloride is irritating to the gastrointestinal tract, even to the extent of causing perforation.

Since the resting membrane potential is related to the ratio of the intracellular to extracellular potassium concentration, hyperkalemia partially depolarizes the cell membrane. Prolonged depolarization impairs membrane excitability and is manifested as weakness, which may progress to flaccid paralysis and hypoventilation if the respiratory muscles are involved. The most serious effect of hyperkalemia is cardiac toxicity. Electrocardiographic changes include increased T-wave amplitude, prolonged PR interval and QRS duration, and atrioventricular conduction delay, which may eventually lead to ventricular fibrillation or asystole (Kokko 1996).

In general, a moderate increase in dietary potassium intake is not likely to produce any toxic effects, unless its renal excretion is severely compromised. The regulatory mechanisms of the human body have evolved to save sodium and actively excrete potassium; the current high dietary sodium intake, especially in ratio to potassium, goes against this adaptation and is much more likely to cause problems (Tobian 1997).

2.4 PLANT STEROLS

Sterols are essential components of all cell membranes both in animals and plants. The sterol ring is common to all sterols; the differences are in the side chain (FIGURE 3). Cholesterol is the exclusive sterol in mammalian cells, whereas numerous other sterols are

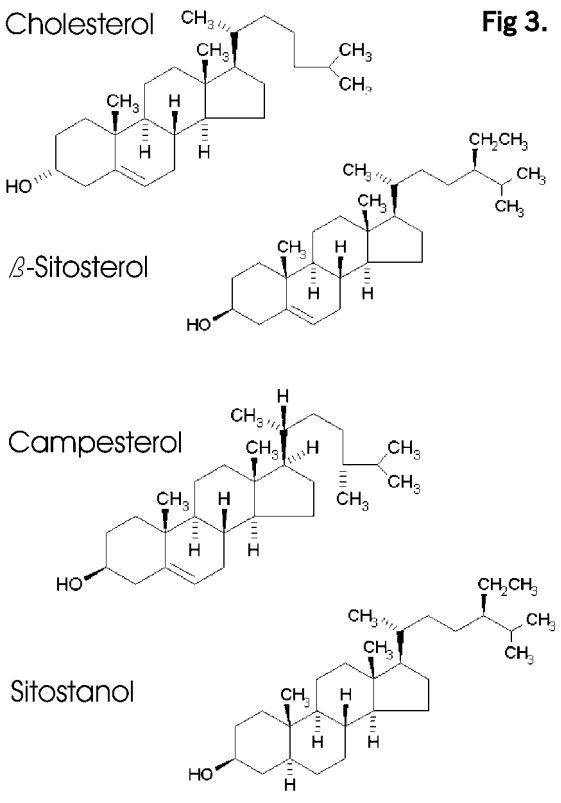


Fig 3.

produced by plants (for review, see Jones et al. 1997). Over 40 plant sterols (or phytosterols) have been identified, but β-sitosterol, campesterol and stigmasterol are the most abundant in nature. Although structurally similar to cholesterol, plant sterols are not synthesized by the human body. Stanols are saturated derivatives of sterols, and less common in nature than sterols.

The dietary intake of plant sterols is an estimated 100–200 mg/d and subject to great variation, while the intake of cholesterol is about 500 mg/d; vegetable oils and cereal products are the most important sources of plant sterols in the average Western diet (Jones et al. 1997). Plant sterols and stanols especially are very poorly absorbed from the intestine.

The specific sterols and stanols that are currently incorporated into foods are extracted from soybean oil or tall oil, and usually esterified to fatty acids to increase lipid solubility (Lichtenstein & Deckelbaum 2001). In the following, the term "plant sterol" is used for all plant-derived sterols and stanols, and "natural plant sterol" refers specifically to the non-esterified forms.

2.4.1 PLANT STEROLS AND SERUM LIPIDS

The cholesterol-lowering properties of plant sterols have been known since the 1950's (Pollak 1952), but almost forgotten until the emergence of the functional food boom of the last decade, which created new financial and scientific interest in this area. Nowadays, the serum cholesterol-lowering effect of plant sterols in patients with mild hypercholesterolemia is well established (Law 2000), and food products containing these compounds have been widely accepted as part of a healthy antiatherogenic diet (Lichtenstein & Deckelbaum 2001). Plant sterols and stanols have also proven effective during low-fat and low-cholesterol diets (Hallikainen & Uusitupa 1999; Hallikainen et al. 2000), and even in combination with lipid lowering drugs (Blair et al. 2000).

Inhibition of cholesterol absorption

Plant sterols lower serum cholesterol levels mainly by reducing the absorption of cholesterol from the intestine; they do this by competing for the limited space and reducing the solubility of cholesterol in mixed micelles formed by the action of bile salts, cholesterol and fatty acids (for review, see Lu et al. 2001). These micelles deliver the mixtures of lipids for absorption into the surface of the enterocyte brush border. Cholesterol appears to be specifically removed from the micelles as part of the absorption process, while non-cholesterol sterols such as sitosterol, even if presented at the brush border membrane, are largely excluded from entry and esterification (Lu et al. 2001). A small part of plant sterols, however, is absorbed and this fraction appears to be proportionate to cholesterol absorption efficiency; therefore, the serum concentrations of plant sterols can be used as markers of intestinal cholesterol absorption (Tilvis & Miettinen 1986; Miettinen et al. 1990). The exact mechanism of the absorption of cholesterol is still unknown; however, it is not simple diffusion as previously thought, but an energy-dependent, protein-mediated process (Lu et al. 2001). Probably it is controlled by nuclear hormone receptors, which regulate the expression of transporter proteins such as the scavenger receptor class B type 1 (SR-B1) (Hauser et al. 1998) and possibly also by the ATP-binding cassette, ABCA1 (Chen 2001) on the enterocyte

membrane. After absorption, the free sterols and fatty acids are esterified in the enterocytes by the action of acyl-CoA:cholesterol acyl-transferace (ACAT), packaged with triglycerides, phospholipids and apo-B48 into chylomicrons, and finally secreted to the lymphatic channels to be transported into the peripheral circulation (Lu et al. 2001). Only a small part, generally less than 5–10%, of the ingested plant sterols are present at these later stages of absorption, but it is possible that they affect some of the enzymes at this level as well (Ling & Jones 1995).

Effects on cholesterol metabolism

Endogenous synthesis of cholesterol is stimulated as a compensatory response to cholesterol malabsorption and depletion of the hepatic cholesterol pool by plant sterols (Vanhanen et al. 1993; Jones et al. 2000). However, the increased synthesis is not able to fully compensate for the intestinal loss, and so serum LDL cholesterol concentrations decrease: at the recommended dose of 2 g/d the decrease in LDL cholesterol is usually about 10% in mildly hypercholesterolemic subjects (Law 2000; Lichtenstein & Deckelbaum 2001). Lipoprotein kinetic studies have associated the decreased LDL cholesterol levels with a decreased production rate of LDL apo-B; the general lack of effect of plant sterols on HDL cholesterol levels was reflected in essentially no change in the kinetic parameters of HDL apo-A1 (Gylling & Miettinen 1994).

FIGURE 4 shows a simplified scheme of the cholesterol synthesis pathway, highlighting the immediate precursor sterols, which can be measured by gas liquid chromatography from serum sterol extract. Δ^8-cholestenol, desmosterol, lathosterol and, less consistently, squalene, have been found to reflect cholesterol synthesis (Miettinen 1970; Miettinen et al. 1990), and their ratios to serum cholesterol are generally used to evaluate the effects of plant sterols and other dietary and drug interventions on the endogenous synthesis of cholesterol (Miettinen et al. 1995; Miettinen et al. 1998; Gylling et al. 1999; Relas et al. 2000).

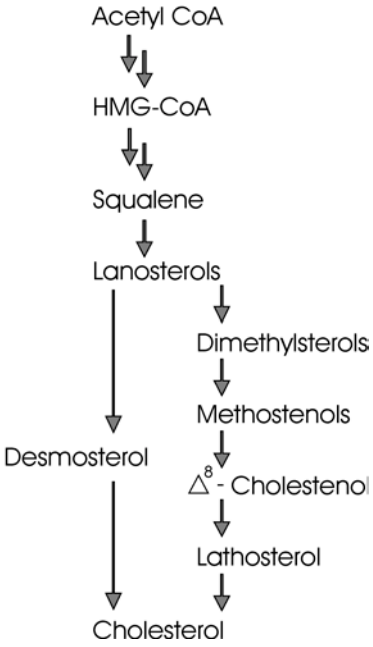


Fig 4. Synthesis of cholesterol (adapted from Relas 2000)

2.4.2 PLANT STEROLS AND BLOOD PRESSURE

Plant sterols are not commonly thought to affect blood pressure in any way, and no data about studies examining this question in either animals or humans appears to be available. However, through their cholesterol-lowering action, plant sterols might be speculated to have indirect long-term effects on blood pressure. For example, there is considerable experimental and clinical evidence that hyperlipidemia can induce glomerular

injury, and that treatment of hyperlipidemia can reduce this damage (Kasiske et al. 1988; Kasiske et al. 1990; Greco & Breyer 1997); renal dysfunction, in turn, will almost inevitably produce hypertension (Hall et al. 1999; Adamczak et al. 2002) . Furthermore, hyperlipidemia is known to induce endothelial dysfunction (Ross 1999; Kinlay et al. 2001), and even short-term intensive cholesterol reduction lowers blood pressure and large artery stiffness (Ferrier et al. 2002). The blood pressure lowering effect of statins, though, may also be related to their direct interaction with endothelial function or angiotensin II receptors (Borghi et al. 2001), and there is no direct evidence that plants sterols would produce a similar effect. In an experimental study, β-sitosterol did not have any relaxing effect on mesenteric artery *in vitro* (Nevala et al. 1998).

2.4.3 PLANT STEROLS AND OBESITY

Although obesity and serum lipids are closely related, very little is known about the effects of plant sterols or any other lipid-lowering therapies on body fat. In experimental studies, decrease in body weight is usually considered a sign of toxicity, and no such effect has been observed with up to 8.1% (w/w) dietary levels of plant sterols (Hepburn et al. 1999). On the other hand, plant sterols are usually supplemented with fat, which may even increase energy intake and fat deposition. In randomized clinical studies that have reported body weights, no significant effect attributable to plant sterols or stanols have been found (Miettinen et al. 1995; Hallikainen & Uusitupa 1999; Hallikainen et al. 2000; Davidson et al. 2001; Mensink et al. 2002). Effects of plant sterols in obese subjects in particular have not been studied. Recently, however, it was shown that sitosterol inhibits cell growth and triglyceride accumulation in 3T3-L1 cells, which is an *in vitro* model for obesity research (Awad et al. 2000). This suggests that at least the absorbed and circulating part of plant sterols could play a role in the control of adiposity.

2.4.4 ADVERSE EFFECTS OF PLANT STEROLS

Based on extensive safety evaluation studies, both plant sterols and stanols are generally recognized as safe (for review, see Plat et al. 2000). However, while plant sterols inhibit the absorption of cholesterol, they may also interfere with the absorption of other lipid soluble components, such as vitamins and antioxidants. Most studies have found that plant sterols lower plasma levels of α-carotene, β-carotene and lycopene, but not the vitamins A, D or K (Hallikainen et al. 1999; Plat et al. 2000). After lipid standardization, even the carotene-lowering effect has often disappeared; the same applies for plasma tocopherols as well (Hallikainen et al. 2000; Plat et al. 2000). The clinical importance of these changes is not known; the observed reductions have not been dose-dependent for either the carotenoids or the tocopherols, and their plasma levels have always been within the normal ranges.

Earlier findings in fish have suggested that plant sterols may have estrogenic activity and effects on the reproductive system (MacLatchy & Van Der Kraak 1995; Mellanen et al. 1996). In recent studies, however, no evidence of any such effects of even very large doses have been found in other species in experimental (Baker et al. 1999; Waalkens-Beerendsen et al. 1999) or clinical conditions (Ayesh et al. 1999).

3 AIMS OF THE STUDY

The present series of experiments was designed to examine the possible benefits of the use of a dietary combination of mineral nutrients and natural plant sterols to serum lipids, blood pressure and body weight, to find an optimal combination of these components, and to provide some insight into the mechanisms of the effects observed in an experimental model of obesity and the metabolic syndrome, the obese Zucker rat.

More specifically, the aims of the experiments were:

1. To compare the effects of a combination of plant sterols and the mineral nutrients calcium, magnesium and potassium, with the effects of the plant sterols alone or the minerals alone on serum cholesterol and body weight during a high-fat and high-cholesterol diet.

2. To compare the effects of a combination of plant sterols and the *divalent* cations calcium and magnesium with the effects of a combination of plant sterols and the *monovalent* cations sodium and potassium on serum cholesterol and body weight during a high-fat and high-cholesterol diet.

3. To evaluate the effects of different dietary calcium levels, and interaction between calcium and plant sterols, on serum lipid profile and absorption and synthesis of cholesterol during both low- and high-fat diets.

4. To examine the long-term effects of the combination of plant sterols and the mineral nutrients calcium, magnesium and potassium on serum lipids, blood pressure, endothelium-mediated vasodilation, and survival in the obese Zucker rat.

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS

Female obese Zucker rats were purchased from Harlan (Oxon, UK). The rats were housed in groups of three or four in plastic cages in an animal laboratory at a room temperature of 22–24°C with a 12-hour light/dark cycle. After an initial adaptation period of one or two weeks, the 8–13 week-old rats were matched for body weight and serum cholesterol and divided into experimental groups to receive different diets. They had free access to the food and tap water at all times.

4.2 DIETS

The experimental diets were based on a commercial low-mineral rat chow (Altromin, Lage, Germany) into which cholesterol, unsalted butter, sodium, calcium, magnesium and/or potassium salts (or a commercially available mineral salt), and a pine oil-derived plant sterol mixture (UPM-Kymmene, Lappeenranta, Finland) were added. The chemical composition of the powder was as follows: β-sitosterol 79.2%, sitostanol 11.4%, campesterol 7.5%, campestanol 1.2%, cycloartenolol 0.5%, and δ-7-avenasterol 0.2%. The different components were mixed with powdered rat chow, and moistened with water using an industrial dough mixer. The chow preparations were then packed in one-day portions and stored at –20 °C.

The exact compositions of the diets in each study are given in the original publications *I–IV*. A summary of the experimental design of the studies is presented in TABLE 4.

4.3 MEASUREMENT OF BLOOD PRESSURE AND HEART RATE

4.3.1 TAIL-CUFF METHOD

Systolic blood pressure and heart rate of the pretrained rats were measured using a tail-cuff analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, U.S.A.). The digital values for systolic blood pressure and heart rate were evaluated automatically from the analog data by a microprocessor. Before the measurements the rats were warmed for 10–20 minutes at 30°C to make the pulsations of the tail artery detectable. Values for systolic blood pressure and heart rate were obtained by averaging readings from three to five measurements.

4.3.2 RADIOTELEMETRY

The Dataquest IV telemetry system (Data Sciences International. St. Paul, MN, USA) was used for measurements of systolic, diastolic and mean arterial pressure, heart rate and locomotor activity, as described previously (Brockway et al. 1991). Twelve-week-old rats were anesthetized with midazolam (5 mg/kg i.p.), fentanyl (0.3 mg/kg i.p.) and fluanisone (10 mg/kg i.p.), and the flexible catheter of the transmitter was surgically secured in the abdominal aorta just below the renal arteries, pointing against the blood flow. The transmitter was sutured to the abdominal wall. The rats were given buprenorfin (0.1 mg/kg s.c.) for the firs two postoperative days. They were housed in individual cages after the operation and were allowed to stabilize for two weeks. The rats were unrestrained and free to move within their cages at all times. The data were sampled every five minutes for ten seconds for each animal. The values for systolic, mean and diastolic blood pressure, heart rate and activity were calculated using the Dataquest software. Mean values were calculated for intervals of 60 minutes for observation of diurnal patterns and for intervals of 12 hours to obtain day- and nighttime curves for the whole four-week period.

4.4 METABOLIC STUDIES

During the last week of each study, the rats were housed individually in metabolic cages. Food intake was measured by weighing the food containers before and after the 24-hour period. Urine was also collected over 24 hours, its volume was measured, and samples were stored at –30°C until analyzed.

4.5 BLOOD SAMPLES AND TISSUE PREPARATION

Before and during the course of the experiments, blood samples (~0.1 ml) from a superficial vein of the foot were taken into capillary tubes for serum cholesterol measurements. At the end of the experiments, the rats anesthetized with a gas mixture containing 70% CO_2 and 30% O_2 (AGA, Riihimäki, Finland) and killed by decapitation. Blood was collected into chilled tubes on ice. After centrifugation, the serum samples were stored at –80°C. The heart was excised and washed with ice-cold saline, the great vessels, atria and the free wall of the left ventricle were dissected, and the left ventricular mass was measured. Kidneys, liver, spleen, thymus, uterus, ovaries, adrenal glands, and forebrain were dissected, washed with saline, blotted dry and weighed. The ratios of left ventricle, kidney and liver weights to skeletal body weight were used as indices to estimate hypertrophy of these organs. The left tibia was excised, carefully cleaned of adherent tissue, and measured using a micrometer, and the length of the tibia was used to estimate the lean or "skeletal" body weight of the obese rats, as suggested by Zucker (1965).

4.6 ARTERIAL RESPONSES

Immediately after decapitation, 3-mm-long standard sections of the superior mesenteric artery, 3 mm distal from the artery-aorta junction, were cut. The rings were placed between stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4), and aerated with 95% O_2 and 5% CO_2. The rings were

Table 4. Summary of the experimental design of the studies

Study (substudy)	Diet components (w/w) Treatment period	Measurements
I (1)	Low-fat control Atherogenic (18% butter, 1% chol, 2.7% Na) + 1% plant sterols + minerals (1.9% K, 0.16% Mg, 2.9% Ca) + plant sterols and the minerals 7 weeks	Serum cholesterol Serum and liver sterols Serum glucose and insulin Body weight Heart, kidney, liver weights Food intake Urine minerals
I (2)	High-fat (18% butter, 1% cholesterol) + 1% plant sterols + 1.8% Na and 1.6% K + 1.8% Ca and 1.6% Mg 4 weeks	
II (1)	Low-fat + 0, 0.5, 1, 2, and 4% cholesterol 2 weeks	Serum cholesterol
II (2)	Low-fat + 1% cholesterol + 0, 0.5, 1, 2, and 4% plant sterols 4 weeks	Organ weights Blood count
II (3)	Low-fat, 1% cholesterol + 0.2, 0.8 and 2.1% calcium 8 weeks	Serum total, LDL and HDL cholesterols, triglycerides, non-cholesterol sterols
II (4)	Low-fat, 1% cholesterol + 0.2 and 2.1% calcium, 1% plant sterols 8 weeks	
III	High-fat (18% butter, 1% cholesterol) + 0.2, 0.8 and 2.1% Ca + 0.2, 0.8 and 2.1% Ca, 1% plant sterols 8 weeks	Serum total, LDL and HDL cholesterol, triglycerides, non-cholesterol sterols Food intake, body weight
IV	Low-fat control Atherogenic (18% butter, 1% chol, 2.4% Na) + plant sterols and minerals (1.2% Na, 1.2% K, 1.7% Ca, 0.1% Mg) 4 weeks – 2 years	Serum cholesterols Blood pressure Arterial responses *in vitro* Food intake, body weight, urine minerals and protein Long-term survival

equilibrated for 20 minutes at +37°C with a resting tension of 1.0 g. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FTO3C transducer, Model 7C8 Polygraph, Grass Instrument Co., Quincy, MA, U.S.A.). The contractile concentration-curves to noradrenaline and potassium chloride, and the relaxation concentration-curves after 1 μM noradrenaline-induced pre-contraction to acetylcholine (to test endothelium-dependent relaxation) and sodium nitroprusside (for endothelium-independent relaxation) were determined as described previously (Mervaala et al. 1994).

4.7 TISSUE MORPHOLOGY

In the long-term survival experiment (*IV*), rats that were either paralyzed or otherwise clearly ill, i.e. stopped moving and eating, were killed by decapitation and coded as dead on the same day, according to the ethical guidelines of the Animal Experimentation Committee. From these few rats, heart and kidney tissue samples were taken. After excision, the samples were fixed in phosphate buffered formaline and then prepared following standard laboratory procedures, dehydrated, embedded in paraffin and cut into 3 μm sections, stained with Masson's trichrome, and examined and photographed under light microscopy.

4.8 BIOCHEMICAL ANALYSES

4.8.1 SERUM LIPIDS, GLUCOSE AND INSULIN CONCENTRATIONS

Serum cholesterol and glucose were measured during the experiments using an automatic analyzer (Reflotron®, Boehringer Mannheim, Germany). Serum lipids from the samples taken at the end of the experiments were analyzed by an accredited laboratory, United Laboratories Ltd., Helsinki, Finland (Hitachi 912 Automatic Analyzer, Hitachi Ltd. Tokyo, Japan). Serum total cholesterol was determined with an enzymatic method (Boehringer Mannheim CHOD-PAP-method), serum HDL cholesterol with an enzymatic direct method (Boehringer Mannheim HDL-Chol Plus), serum LDL cholesterol with an enzymatic direct method (Boehringer Mannheim LDL-Chol Plus), and serum triglycerides with an enzymatic method (Boehringer Mannheim CHOD-PAP-method). Serum insulin was measured using a commercial radio-immunoassay (Incstar Corp., Stillwater, MN, USA).

4.8.2 SERUM CHOLESTEROL PRECURSORS AND PLANT STEROLS

The concentrations of serum non-cholesterol sterols were analyzed by gas chromatography mass spectrometry using a Hewlett-Packard (HP) 5890 gas chromatograph equipped with an NB-54 fused-silica capillary column (15 m×0.20 mm I.D.; Nordion, Helsinki, Finland) and interfaced with an HP 5970A mass spectrometry detector operating in electron impact mode (70 eV). The column oven was programmed from 230 °C to 285 °C at 10 °C/min and injector and detector were at 285 °C. The lipids from serum samples (200 μl) were extracted with chloroform/methanol (2:1) and trans-esterified with sodium methoxide. The released free sterols were trimethylsilylated as described previously (Gylling et al. 1999) and quantified by single ion monitoring technique using m/z 129 (cholesterol, campesterol and β-sitosterol),

m/z 215 (β-sitostanol), m/z 343 (desmosterol), m/z 255 (lathosterol) and m/z 217 (5-α-cholestane, internal standard) as selected ions.

4.8.3 URINE ANALYSES

The concentrations of the mineral elements and albumin and creatinine in urine were analyzed by the United Laboratories (for Na, K and Ca, BM/Hitachi 912 ion selective electrode, Boehringer-Mannheim, Germany; for Mg, Hitachi 180-80 Polarized Zeeman Atomic absorption spectrophotometer). Urinary excretion of the end products of nitric oxide metabolism, nitrate and nitrite, were measured using a colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). Total protein concentration in urine was determined by the method of Lowry et al. (1951).

4.9 COMPOUNDS

The following compounds were used: cholesterol, calcium carbonate, calcium chloride, potassium chloride, magnesium oxide, acetylcholine chloride, and noradrenaline bitartrate (Sigma Chemical Co., St. Louis, MO, USA); sodium chloride, calcium chloride, and calcium carbonate (University Pharmacy, Helsinki, Finland); sodium nitroprusside (Hoffman La Roche, Basel, Switzerland), mineral salt (Pansalt®, Oriola, Espoo, Finland), plant sterol mixture (UPM-Kymmene, Lappeenranta, Finland), midazolam (Dormicum®, Hoffman La Roche, Basel, Switzerland), fentanyl-fluanisone (Hypnorm®, Janssen Pharmaceutica, Beerse, Belgium) and buprenorfin (Temgesic®, Reckitt & Colman, Hull, UK). All compounds that were given to the animals subcutaneously or intraperitoneally were dissolved in 0.9% NaCl solution. The stock solutions of the compounds used in *in vitro* studies were dissolved in distilled water. All solutions were freshly prepared before use and protected from light.

4.10 STATISTICAL ANALYSES

Statistical analysis was carried out by one-way analysis of variance (ANOVA) supported by either Tukey's test or Fisher's Least Significant Difference test. Data for multiple observations over time were analyzed by two-way ANOVA with repeated measures for overall treatment effect, and Tukey's test was used for multiple pair wise comparisons of treatment groups at different times. Data for dose-response relationships were analyzed by calculating Pearson correlation coefficients, followed by a two-tailed significance test. Differences between means that had $p < 0.05$ were considered significant. The data were analyzed with SYSTAT Statistical Software (SYSTAT Inc., Evanston, IL, U.S.A.). The results are expressed as means ± S.E.M.

4.11 ETHICS

The procedures and protocols of the studies were in accord to the ethical guidelines of the Institute of Biomedicine, University of Helsinki, and approved by the Animal Experimentation Committee of University of Helsinki, or by the Provincial State Office of Southern Finland.

5 RESULTS

5.1 SERUM LIPIDS

5.1.1 EFFECTS OF CALCIUM

In the obese Zucker rats receiving a low-fat diet with 1% cholesterol (*II*), increases in dietary calcium intake from 0.2 to 0.8, and further to 2.1% (w/w) dose-dependently lowered serum total cholesterol, LDL cholesterol, and triglyceride concentrations, and raised HDL cholesterol concentration, HDL cholesterol to total cholesterol ratio, and HDL to LDL cholesterol ratio. Calcium also dose-dependently increased the serum cholesterol precursors (desmosterol and lathosterol) to cholesterol ratios as well as the plant sterols (campesterol, campestanol, sitosterol, and sitostanol) to cholesterol ratios.

In the obese Zucker rats receiving a high-fat diet with 1% cholesterol (*III*), increases in dietary calcium intake dose-dependently decreased serum total cholesterol and LDL cholesterol concentrations. Serum HDL cholesterol also tended to decrease, but HDL to LDL cholesterol ratio was increased. There was no significant correlation between dietary calcium and serum triglyceride levels. Increases in calcium intake dose-dependently increased serum desmosterol to cholesterol and lathosterol to cholesterol ratios, but did not significantly affect serum campesterol to cholesterol or sitosterol to cholesterol ratios.

5.1.2 EFFECTS OF PLANT STEROLS

Dietary plant sterol supplementation dose-dependently decreased serum cholesterol in the obese Zucker rats receiving a low-fat diet with 1% cholesterol (*II*). A plant sterol content of 1% was required for a statistically significant effect, and 4% completely prevented the diet-induced increase in serum cholesterol. The low-calcium diet with 1% of plant sterols produced approximately 30% decrease in serum total cholesterol, 60% decrease in LDL cholesterol and 70% increase in HDL cholesterol, but no significant change in serum triglycerides. Plant sterols increased serum desmosterol to cholesterol and lathosterol to cholesterol ratios as well as serum plant sterols to cholesterol ratios.

As compared with a diet containing 18% of butter and 1% of cholesterol, the 1% plant sterol supplementation lowered serum total cholesterol by approximately 30% (*I*) or 40% (*III*); LDL cholesterol was lowered by about 80% and HDL cholesterol by 60%; triglycerides were increased by about 60% and HDL to LDL cholesterol ratio increased by 170%. Plant sterols supplementation tended to increase

serum desmosterol to cholesterol and lathosterol to cholesterol ratios, and serum plant sterols to cholesterol ratios were three to four fold increased in all plant sterols supplemented groups.

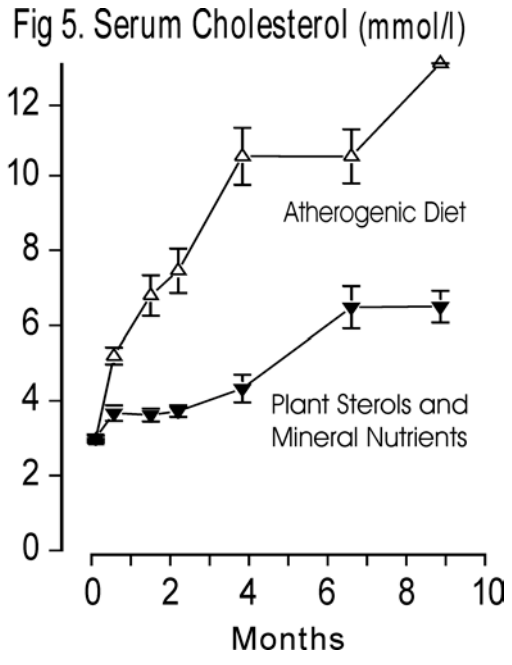
5.1.3 EFFECTS OF COMBINATIONS OF MINERALS AND PLANT STEROLS

As compared with a low-fat, low-calcium diet with 1% cholesterol (*II*), the combination of 2.1% dietary calcium and 1% plant sterols produced about 20% decrease in serum total cholesterol, 60% decrease in LDL cholesterol, 180% increase in HDL cholesterol and 850% increase in HDL to LDL cholesterol ratio. Serum desmosterol to cholesterol ratio and the plant sterols to cholesterol ratios were significantly increased by the plant sterols and calcium supplementation.

As compared with a high-fat, low calcium diet with 1% cholesterol (*III*), the combination of 2.1% dietary calcium and 1% plant sterols produced about 70% decrease in serum total cholesterol, 80% decrease in LDL cholesterol, 60% decrease in HDL cholesterol and 70% increase in HDL to LDL cholesterol ratio. Both serum desmosterol to cholesterol and lathosterol to cholesterol ratios, and the plant sterols to cholesterol ratios were significantly increased by the plant sterols and calcium supplementation.

During a diet containing 18% of butter and 1% of cholesterol (*I*), a supplementary combination of 5.5% of sodium and potassium salts and 1% of plant sterols produced about 20–30% lower serum total cholesterol level, the same as that produced by plant sterols alone. In contrast, on a combination diet of 5.5% of calcium and magnesium salts with 1% plant sterols serum total cholesterol was 50–70% lower, i.e. the diet-induced rise of the cholesterol levels was completely prevented.

In the obese Zucker rats fed an atherogenic diet containing 18% of butter, 1% cholesterol and 6% of common salt (w/w), dietary combination of plant sterols with calcium, magnesium and potassium salts, which partly replaced the common salt (*IV*), also nearly completely prevented the rise in serum total cholesterol. The ten-fold rise in LDL cholesterol was partially prevented, along with an insignificant decrease in HDL cholesterol and rise in triglyceride levels; HDL to LDL cholesterol ratio was, however, markedly increased. In the long-term experiment, the total cholesterol levels tended to rise with advancing age in both groups, but the values in the plant sterols and mineral nutrients supplemented group were still after nine months less than half of those in the atherogenic diet group ($p < 0.001$, $n = 10–15$ per group) (FIGURE 5, unpublished data).



5.2 BLOOD PRESSURE AND ARTERIAL TONE (*IV*)

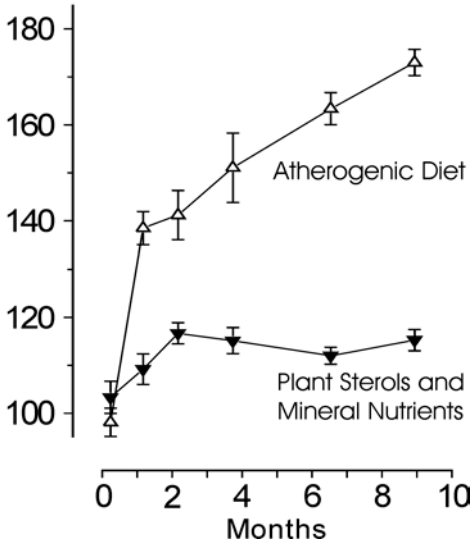
The blood pressure of the rats on the atherogenic diet with 18% of butter, 1% of cholesterol and 6% of common salt (w/w), increased steadily during the four-week experiment. The effect was most pronounced in nighttime systolic pressure, but even daytime and mean arterial pressures were significantly elevated. The 1% plant sterols and 3% mineral nutrients supplemented, sodium-reduced diet completely prevented this hypertensive effect of the atherogenic diet. The pressure readings of this group remained on the same low level as those of the low-fat, low-salt diet fed control group, with no significant rise over time.

There was considerable diurnal variation in the systolic and mean arterial pressures, heart rate and activity. All of these parameters showed higher readings at nighttime, when the rats were active and ate most of their food. There were no significant changes in the heart rate or activity levels over time during the four-week experimental period. However, heart rate was consistently lower at all times in the plant sterols and minerals supplemented diet group as compared with the other groups.

The endothelium-dependent relaxation responses to acetylcholine were significantly improved in the plant sterols and minerals supplemented diet group as compared with the atherogenic and control diet groups. The endothelium-independent relaxations to sodium nitroprusside were not significantly affected by the diet changes. There were no significant differences in the contractile responses to noradrenaline or potassium chloride between the groups.

In the long-term study, blood pressure was measured by the tail-cuff method until about nine months when many of the rats on the atherogenic diet had already died. There was a continuous rise of systolic blood pressure up to 180 mmHg in the atherogenic diet group, while the plant sterols and mineral nutrients supplemented group remained at the normal 110–120 mmHg level ($p < 0.001$, $n = 10–15$ per group) (FIGURE 6, unpublished data).

Fig 6. Systolic Blood Pressure (mmHg)



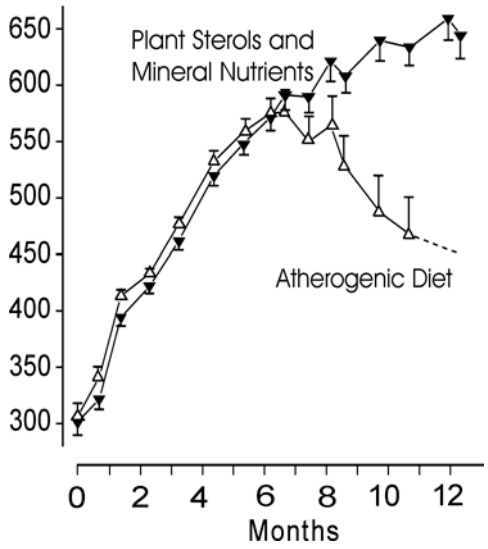
5.3 BODY WEIGHT, GLUCOSE AND INSULIN

The 2.1% calcium supplemented diets in study *III* resulted in 10–20% lower end weights as compared with the diets of lower calcium contents, in spite of equal or higher food intake. Plant sterols alone or in combination with sodium and potassium had no significant effect on body weight gain (*I–II*) or food intake. Combination of plant sterols with calcium (*III*), or calcium and magnesium (*I*), or calcium, magnesium and potassium salts (*IV*) markedly reduced the development of

obesity in Zucker rats during high-fat diets. Food and energy intakes in the calcium-supplemented groups were the same as or higher than those for the other groups in all these studies.

In the long-term study, body weight in the plant sterols and mineral nutrients supplemented group increased steadily, however, remaining slightly but significantly lower than in the atherogenic diet group until about five or six months, after which the rats on the atherogenic diet started to lose weight and die (FIGURE 7, unpublished data).

Fig 7. Body Weight (g)

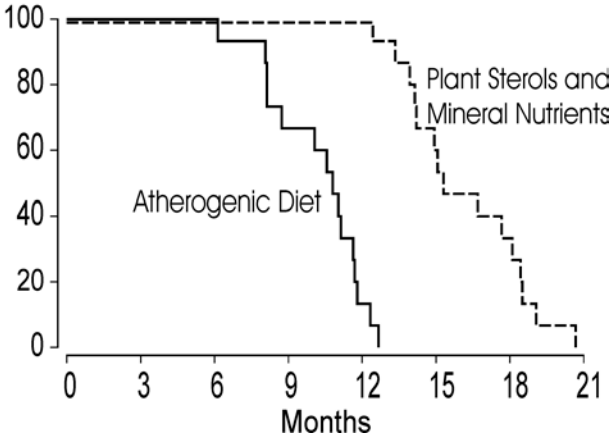


Serum glucose levels of the obese Zucker rats were constantly about 7–8 mmol/l and did not change significantly during any of the dietary treatments. However, in study *I*, where also insulin levels were measured, the serum insulin to glucose ratio after seven-week treatment (µg/mmol, unpublished data) was 5.6 ± 0.7 in the low-fat control group, 3.3 ± 0.4 in the atherogenic diet group, 3.8 ± 0.4 in the plant sterols supplemented group, 2.9 ± 0.4 in the mineral nutrients supplemented group, and 1.9 ± 0.2* in the plant sterols and minerals supplemented group (*$p < 0.05$ *vs.* other groups). In the second part of study *I* the serum insulin to glucose ratio after four-week treatment (µg/mmol, unpublished data) was 10.5 ± 1.8 in the high-fat control group, 9.2 ± 1.6 with plant sterols supplementation alone, 5.3 ± 0.5* with plant sterols, sodium and potassium, and 4.3 ± 0.7* with plant sterols, calcium and magnesium (*$p < 0.05$ *vs.* control group and plant sterols alone).

5.4 SURVIVAL AND TISSUE MORPHOLOGY (*IV*)

The plant sterols and mineral nutrients supplemented diet extended the average life span of the rats by 60% as compared with the atherogenic diet ($p < 0.001$, $n = 15$ per group at start) (FIGURE 8, *IV*). Most of the deaths in both diet groups were sudden, and no samples could be obtained from dead animals, but in those few rats on the atherogenic diet that had to be killed, atherosclerotic lesions, thromboses in coronary arteries and myocardial infarctions were observed. In the kidneys, intimal thickening and occlusion of arteries were seen, and most glomeruli were destroyed.

Fig 8. Cumulative proportion of surviving rats (%)



6 DISCUSSION

Dietary modifications to lower the risk of cardiovascular diseases are increasingly recommended for the general population, and particularly for individuals with high serum cholesterol, hypertension or obesity. Products containing plant sterols and stanols are already marketed for lowering of serum cholesterol. Potassium and magnesium enriched mineral salts are also available, and replacement of common salt by such mineral salts in the diet has been shown to lower blood pressure. Calcium, besides being antihypertensive, may also have beneficial effects on serum lipids. Concurrent implementation of these different dietary approaches may enhance their effects and even produce additional benefits (Karppanen et al. 2000), but has not been studied previously. Therefore, the present study investigated the effects of a combination of dietary plant sterols and mineral nutrients on serum lipids, blood pressure and obesity using Zucker rat as the experimental model.

6.1 METHODOLOGICAL ASPECTS

The *fa/fa* mutation of the genetically obese Zucker rat was first described by Zucker & Zucker (1961) and has thereafter been extensively characterized to exhibit hyperlipidemia, insulin resistance and renal injury as recessive traits (Zucker 1965; Kasiske et al. 1992). The mutation has recently been localized in the extracellular domain of the leptin receptor (Chua et al. 1996; Phillips et al. 1996). Lack of leptin control of satiety leads to hyperphagia, which produces hyperinsulinemia, which in turn upregulates transcription factors that stimulate lipogenesis. Besides growth of adipose tissue, this causes ectopic deposition of triglycerides in other tissues such as heart, kidney and pancreatic β-cells, where nonoxidative metabolism of fatty acids produces damaging compounds, resulting in functional impairment and apoptosis (for review, see Unger & Orci 2001). This phenomenon, referred to as lipotoxicity, is believed to cause the common complications of obesity, insulin resistance, cardiovascular disease and diabetes; therefore, the obese Zucker rat is considered a useful experimental model of the human metabolic syndrome (Kasiske et al. 1992; Unger & Orci 2001).

Comparison of the presumably monogenic disorder of the rat with the polygenic and environmentally induced human counterpart is certainly complex in many ways. Lack of leptin action, although such mutations have been reported (Clement et al. 1998), is not a major causative factor in human obesity (Rolland et al. 1998), and the whole concept of lipotoxicity in humans in this context is somewhat controversial. However, regardless of the

cause, disproportion between energy intake and consumption is the common element in the development of obesity in both species, and the array of complications is surprisingly similar. Thus, while more specific models may exist for separate studies on hyperlipidemia, hypertension, and obesity, the obese Zucker rat is one of the few (Chen & Garg 1999) that seems suitable for examining the prevention and treatment of the whole syndrome.

In the present study, most serum lipid analyses were performed in an accredited commercial biochemistry laboratory. Before and during the experiments, serum total cholesterol was determined by reflotron capillary analysis. This system was developed for screening purposes in humans and has proven fairly fit for that use (Statland 1990). It has not been previously tested for rats, and could thus be unreliable; however, analysis of pooled parallel measurements of the present series of studies showed an excellent correlation between the reflotron and standard laboratory values of serum cholesterol ($r = 0.951$, $p < 0.001$, $n = 104$).

Blood pressure was measured either directly by radiotelemetry or indirectly by the tail-cuff method. Radiotelemetry is a high-fidelity method for 24-hour monitoring of systolic and diastolic blood pressures, heart rate, and activity of freely moving animals for relatively long periods, without the stress of restraints (Bazil et al. 1993). Major limitations of this method, besides its expense, are the need for initial surgery, the possible stress caused by isolation of the animal, and the few months' duration of the transmitter battery. Also the drifts in telemeter settings and sensitivity may cause problems, which makes it necessary to recalibrate the device immediately before implantation (Van Vliet et al. 2000).

The indirect but non-invasive tail-cuff method is a more reasonable means for measurement of blood pressure, if the experiment involves large numbers of animals and/or very long time periods. In this procedure, the rats must be restrained in a heated case to be able to detect the pulsations in the tail artery and obtain decent blood pressure values. This obviously poses a stress factor, which is likely to elevate the blood pressure levels, although in general the correlations with intra-arterial measurements have been good (Bazil et al. 1993). Comparability of the telemetric and tail-cuff values in the obese Zucker rat was also confirmed by pilot measurements outside the present study (unpublished observation). Stress-induced fluctuations can be minimized by pre-training the animals, by performing the measurements regularly by the same person in a peaceful environment, and by averaging readings from several consecutive measurements. One limitation of the tail-cuff method is that it only produces reliable values for systolic blood pressure.

6.2 SERUM LIPIDS

The composition of serum lipids in the obese Zucker rat was strongly dependent on the fat and mineral contents of the diet. Supplementation of a low-mineral diet with cholesterol and butter produced up to 10–20 fold increases in serum total and LDL cholesterols. Without the butter, the increases were less remarkable. A diet containing high levels of cholesterol and saturated fat is a major cause of elevated serum cholesterol also in humans (Sempos et al. 1993; Verschuren et al. 1995). Saturated fats have long been known to

raise serum cholesterol levels, but the mechanism of this effect is still not completely understood; probably the saturated fatty acids inhibit the receptor-mediated uptake of LDL into liver cells, thereby decreasing the clearance of LDL particles from the circulation (Kris-Etherton et al. 1988; Grundy & Denke 1990).

The most important finding of the present study was that supplementation of a high-cholesterol and high-fat atherogenic diet with the combination of plant sterols and the mineral nutrients calcium, magnesium and potassium effectively lowered serum total and LDL cholesterols, and quite clearly extended the life span of the obese Zucker rats. Part of this effect was obviously due to the added plant sterols in the diet. The findings of studies *I–III*, where plant sterols were examined separately, are consistent with previous studies both in animals and in man; they have shown that natural plant sterols, or their chemically modified derivatives, lower serum cholesterol by inhibiting the intestinal absorption of cholesterol (Law 2000; Lichtenstein & Deckelbaum 2001).

However, a significant part of the effect can also be assigned to the increased content of calcium in the diet. Studies *I–III* that examined the effects of different mineral compositions showed that calcium and magnesium were not only able to lower serum cholesterol but also enhanced the effect of plant sterols. In particular, dietary calcium intake alone had an inverse correlation with serum total and LDL cholesterols and, during a low-fat diet, even a positive relation with HDL cholesterol. These results are in agreement with previous studies with calcium supplements in experimental (see TABLE 2 on page 15) as well as clinical studies (TABLE 3 on page 17). In compliance with previously documented methods (Miettinen et al. 1990), analysis of some serum plant sterols and cholesterol precursors suggested that calcium dose-dependently increased the endogenous synthesis of cholesterol, and possibly also its intestinal absorption. This apparently paradoxical effect can be explained by the concept that calcium binds bile acids. Increased fecal loss of bile acids is compensated by increased conversion of cholesterol to bile acids in the liver, which leads to depletion of the hepatic cholesterol pool and eventually to reduction of serum cholesterol levels as well. Thus, the cholesterol-lowering mechanism of calcium would be similar to that of cholestyramine and other resins (Witztum 1996). In theory, the increase in cholesterol precursor levels could also be due to inhibition of the enzymes that convert the precursors to cholesterol, but there is no indication in previous literature of such an action of calcium. Another weakness in this reasoning is that the findings of Miettinen et al. (1990) relate to humans but might not be applicable in rats. Nevertheless, the present conclusion of compensatory increase in cholesterol synthesis is consistent with previous results on treatment with other cholesterol-lowering agents such as plant sterols (Miettinen et al. 1995; Gylling et al. 1999; Jones et al. 2000), neomycin (Miettinen 1982) and bile acid sequestrants (Strandberg et al. 1990; Witztum 1996). In addition to binding bile acids, calcium can also reduce the absorption of fat by forming insoluble soaps with fatty acids (Drenick 1961; Welberg et al. 1994). Decreased absorption of saturated fatty acids could increase the clearance of LDL from the circulation (Kris-Etherton et al. 1988; Grundy & Denke 1990), thereby lowering serum LDL cholesterol levels. The fatty acid binding action of calcium is likely to

contribute to the lowering of serum cholesterol, especially during high intake of saturated fat. Both of these mechanistic views are supported by previous literature: increases in fecal fat and/or bile acid excretion associated with dietary calcium supplementation were reported in several animal as well as human studies (see TABLES 2 and 3).

Increases in dietary calcium intake also enhanced the cholesterol-lowering effect of plant sterols, particularly during a low-fat diet. In the rats fed the high-fat diets, this relationship with calcium was no longer dose-dependent; rather, the effect of plant sterols appeared most pronounced during the low- and moderate-calcium diets. Calcium intake in humans is typically rather low, and intakes as high as in the present study are hardly possible outside experimental conditions. Therefore, it is important to note that already a moderate calcium supplementation with plant sterols lowered serum LDL cholesterol almost as effectively as the high-calcium diet alone, despite the reactive increase of endogenous cholesterol synthesis. The increase in serum triglyceride levels observed in the plant sterol supplemented groups during high-fat diets is clearly a disadvantage of this combination; however, it might be explained by the increased compensatory production of lipid-rich VLDL particles in the liver.

The increases in serum HDL cholesterol (*II*) and HDL to LDL cholesterol ratios (*II–IV*) produced by the combination of calcium and plant sterols are particularly interesting and important, because low HDL cholesterol is an independent risk factor for atherosclerosis and also a marker for the metabolic syndrome (Gotto 2001). Elevated levels of HDL cholesterol alone and in relation to total and LDL cholesterols, accordingly, provide protection against cardiovascular diseases. This effect of HDL has been largely attributed to its role in reverse cholesterol transport, in which cholesterol that has been synthesized or deposited in peripheral tissues is returned to the liver. However, in view of recent research, the connection between lipoproteins is more complex and involves specific protein-receptor interactions and activation of cellular signaling pathways, which regulate proteins involved in platelet activation, thrombosis, cell adhesion, apoptosis and vasomotor functions (O'Connell & Genest 2001). Quite recently, Spieker *et al.* (2002) demonstrated that HDL restores the LDL induced endothelial dysfunction, which is an early event in the atherosclerotic process, in hypercholesterolemic men.

6.3 BLOOD PRESSURE

The high-salt, high-cholesterol and high-fat atherogenic diet markedly increased blood pressures of the obese Zucker rat in both telemetric and tail-cuff measurements. This is in accordance with previous studies pointing to the salt-sensitivity of this model (Reddy & Kotchen 1992; Morgan *et al.* 1995). Excessive intake of dietary sodium is a major cause of hypertension also in humans (Kotchen & McCarron 1998). The plant sterols and mineral nutrients supplemented diet, using a potassium- and magnesium-enriched, sodium-reduced mineral salt instead of common salt, and calcium, completely prevented the development of hypertension in this model. The same mineral salt has a similar effect in another salt-sensitive model, the spontaneously hypertensive rat, even when dietary sodium content is intentionally kept constant (Mervaala *et al.* 1992), and it has been shown to lower

blood pressure in humans as well (Geleijnse et al. 1994; Itoh & Kawasaki 1998; Katz et al. 1999). The antihypertensive effects of potassium and also calcium are well established (Kotchen & McCarron 1998). All these mineral nutrients appear to facilitate renal sodium excretion, although reduction in dietary sodium intake is also important, and may have contributed to the effects observed in the present study as well.

However, not all studies have demonstrated any salt-induced rise in blood pressure in the obese Zucker rat (Pawloski et al. 1992), and a pair-feeding experiment indicated that the obese hypertensive rats retained even less sodium than the normotensive lean controls (Kurtz et al. 1989). Thus, other mechanisms for the hypertensive effect of the atherogenic diet must also be considered. Previously, the development of hypertension in the obese Zucker rat has been associated with the progression of renal damage in aging animals (Kasiske et al. 1992). In the present study, increased albuminuria during the atherogenic diet (*IV*) indicated an early development of renal damage, whereas there was no sign of such damage in the plant sterols and mineral nutrients supplemented diet group. Renal damage is strongly linked to serum lipid profile, in both humans and several animal models; it has been shown that hypercholesterolemia induces glomerulosclerosis and tubulointerstitial damage, and treatment with lipid-lowering drugs improves glomerular filtration rate and reduces albuminuria (Kasiske et al. 1990; Greco & Breyer 1997; Fried et al. 2001). In the present study, the rise of serum cholesterol was almost completely blocked by the plant sterols and mineral nutrients supplementation, which could at least partly explain the protection against renal damage and, consequently, the gradual development of hypertension. This view is supported by the results of the long-term experiment (*IV*), in which the rats on the plant sterols and mineral nutrients supplemented diet lived much longer than the ones on the atherogenic diet, and glomerulosclerosis and necroses were observed in the kidneys of the rats on the atherogenic diet.

Improvement in the endothelium-mediated vasodilation may also partly explain the blood pressure lowering effect of the plant sterols and mineral nutrients supplemented diet. Previous studies have demonstrated that calcium (Pörsti 1991; Mäkynen et al. 1996) as well as potassium- and magnesium-enriched diets (Mervaala et al. 1994; Mäkynen et al. 1995; Tolvanen et al. 1998) improve vascular relaxation and lower blood pressure in different rat models. In the present study (*IV*), the relaxation response to acetylcholine in a muscular conduit artery *in vitro* was markedly increased by the plant sterols and mineral nutrients supplemented diet, even beyond the control diet group, which may have contributed to the protection against the prohypertensive components of the diet. Because the vascular relaxation responses were similar in the atherogenic and control diet groups, the endothelial dysfunction as such is not likely to be the cause of the diet-induced hypertension but rather a character of the species, probably related to obesity and hyperlipidemia (Wu et al. 1996; Duarte et al. 1999). As hyperlipidemia is known to induce endothelial dysfunction (Ross 1999; Kinlay et al. 2001), the antihypertensive effect of the plant sterols and mineral nutrients supplemented diet may also be related to the lipid-lowering action of the combination.

6.4 OBESITY

In agreement with previous reports (Zucker 1965; Kurtz et al. 1989; Kasiske et al. 1992) the Zucker rats developed pronounced obesity with marked accumulation of subcutaneous and abdominal fat during the control and atherogenic diets. Enrichment of the high-fat diets with the plant sterols did not affect weight gain. However, enrichment of the high-fat diets with calcium (and magnesium), both in the absence and in the presence of plant sterols, induced a marked reduction in the development of obesity. In the first study, the compositions and possibly also the taste of the diets were quite different, and the rats might have eaten less and therefore lost weight in the beginning of the study. In the other studies, however, body weight gain was monitored more carefully, and the diets were made as similar as possible so that even the chloride concentrations were adjusted to the same level, and still the difference persisted. These findings are concordant with previous studies of calcium supplements in other rat strains (Fleischman et al. 1967; Yacowitz et al. 1967; Foley et al. 1990; Pörsti et al. 1990; Mäkynen et al. 1996).

In the long-term study, body weight remained slightly lower in the plant sterols and mineral nutrients supplemented rats until about six months, after which the rats on the atherogenic diet started losing weight, obviously due to enervating disease processes. While these rats were dying, the others kept on thriving for another six months and some of them eventually weighed more than 800 g. In this view body weight *per se* did not seem to be a very important factor in longevity. However, the initial better weight control in the plant sterols and mineral nutrients supplemented diet group may partly have delayed the disease processes, thus contributing to the general well-being and reduced mortality. In humans, even a small weight reduction is associated with favorable changes in blood pressure, serum lipids and glucose metabolism and, thus, reduction in the risk of diabetes and heart disease (Anderson & Konz 2001).

The slower increase of body weight in the calcium-supplemented groups of the present studies appeared to be due to reduced accumulation of fat tissue, because the calculated lean or skeletal body weight was not affected, and because urinary creatinine excretion, used as an indicator of muscle catabolism, was not changed. Also the estimated food and energy intakes were equal in the different dietary groups, or in some cases, even higher in the calcium-supplemented groups than in the other groups. Therefore, the reduction in the amount of fat tissue appears to be due to increased loss rather than decreased intake of energy. A plausible mechanism would be the formation of insoluble soaps of fatty acids and the divalent cations calcium and magnesium in the intestine (Drenick 1961; Welberg et al. 1994), leading to reduced absorption of fatty acids so that part of the ingested energy is lost into stools. This view is largely supported by previous studies with calcium supplements (see TABLES 2 and 3), with or without any reported body weight effects; it must be noted that a reduction in fat *absorption* does not necessarily lead to any reduction in fat *accumulation*, if food intake is not controlled. Previous studies have also demonstrated improvements in carbohydrate metabolism by increased intakes of potassium and magnesium (Rapoport & Hurd 1964; McFarland & Carr 1977; Karppanen et al. 1984; Paolisso et al.

1989; Balon et al. 1995). In the present study, the serum insulin to glucose ratio was most markedly decreased in the potassium and magnesium enriched diet groups, suggesting an improvement in insulin sensitivity, although the possibility that this difference was due to the decreased amount of carbohydrates in the diets cannot be excluded. Furthermore, calcium may affect fat metabolism and the control of energy balance. Zemel et al. (2000) have shown that increased circulating levels of the calcitrophic hormones PTH and vitamin D during low-calcium diets increase cellular calcium influx, which in turn stimulates lipogenesis and inhibits lipolysis in adipocytes. Accordingly, increased dietary calcium intake would suppress these calcitrophic hormones and thereby reduce the adipocyte intracellular calcium and lipid storage. The PTH or vitamin D status of the rats was not assessed in the present study. However, in obese mice calcium supplementation has been reported to lower the levels of these hormones and reduce lipid accumulation and body weight (Shi et al. 2001). In humans, high intake of calcium is associated with decreased parathyroid function (McKane et al. 1996), and even acute ingestion of calcium is followed by a significant decrease in serum PTH level (Tohme et al. 1990). Involvement of dietary calcium in the physiological regulation of body weight and adiposity is also indicated by some epidemiological (McCarron et al. 1984; Zemel et al. 2000; Carruth & Skinner 2001) as well as clinical studies (Davies et al. 2000).

6.5 HUMAN IMPLICATIONS

Hyperlipidemia, hypertension, obesity and type II diabetes are strongly interrelated conditions and form a serious health hazard particularly to modern western populations. Dietary and lifestyle factors are most important causes behind these risk factors; therefore, it would be reasonable to look for dietary approaches for their modification. Reduced intake of sodium and increased intakes of calcium, magnesium and potassium have proven antihypertensive in several studies in humans, and are currently recommended for even the general population by health organizations and officials worldwide (Joint National Committee 1997; Kotchen & McCarron 1998; Krauss et al. 2000). Furthermore, calcium is considered important in prevention of some other chronic diseases, primarily osteoporosis (Miller & Anderson 1999). Plant sterols and stanols are also recommended and useful for lowering of elevated blood cholesterol levels (Law 2000; Lichtenstein & Deckelbaum 2001). The DASH study, in particular, has shown the practical relevance of combination diets low in sodium, fat and cholesterol, and high in calcium, magnesium, potassium and plant fiber in treatment of elevated blood pressure (Appel et al. 1997; Sacks et al. 2001) and even in lowering serum lipid levels (Obarzanek et al. 2001). Quite recently, the first clinical trial confirmed the cholesterol-lowering effect of food items prepared according to the principles described in the present experimental study (Tikkanen et al. 2001). Further research in humans would appear warranted to find out if a long-term use of foods enriched with this kind of combination of mineral nutrients and plant sterols is able to produce any of the other beneficial effects that were seen in the obese Zucker rats.

7 SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the effects of mineral nutrients and plant sterols on serum lipids, blood pressure and body weight in an experimental model of obesity and the metabolic syndrome, the Zucker rat. The findings and conclusions are as follows:

1. The combination of plant sterols and the mineral nutrients calcium, magnesium and potassium prevented the diet-induced rise of serum cholesterol more effectively than either the plant sterols or the minerals alone. Dietary supplementation of the mineral nutrients reduced body weight, both in the presence and in the absence of plant sterols.

2. The combination of plant sterols with sodium and potassium did not provide any cholesterol- or body weight-lowering effects beyond that of plant sterols alone, whereas the combination of plant sterols with calcium and magnesium both prevented hypercholesterolemia and markedly reduced the accumulation of excess body fat.

3. Dietary calcium lowered serum total and LDL cholesterols in a dose-dependent manner, and increased the HDL to LDL cholesterol ratio both during low-fat and high-fat diets. These effects were partly enhanced by concurrent plant sterol supplementation. Moreover, high levels of dietary calcium reduced obesity, independently of plant sterols. The effects of calcium are likely to be mediated by inhibition of the intestinal absorption of saturated fat and bile acids.

4. Long-term supplementation of an atherogenic diet with plant sterols and replacement of sodium partly with the minerals calcium, magnesium and potassium effectively prevented the diet-induced increases in total and LDL cholesterol and 24-hour systolic and mean blood pressure, improved endothelium-mediated vasodilation, protected against end-organ damage, and considerably extended the life span of the obese Zucker rats.

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