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UMI
COLON CANCER PROMOTION, INSULIN RESISTANCE AND METABOLIC MEASURES: THE ROLE OF DIETARY CALCIUM, VITAMIN D AND FAT

by

Marie Carmen Chia

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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COLON CANCER PROMOTION, INSULIN RESISTANCE AND METABOLIC MEASURES: THE ROLE OF DIETARY CALCIUM, VITAMIN D AND FAT

Master of Science, 1999
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ABSTRACT

McKeown-Eyssen and Giovannucci suggested that hyperinsulinemia and/or energy availability may be important in both insulin resistance (IR) and colorectal cancer (CRC). In this thesis, it was hypothesized, first, that dietary calcium effects measures of CRC, IR and energy availability to the same extent. Results demonstrated that vitamin D had no effect on these measures and that elevated calcium was related to reduced CRC promotion and weight gain and increased fecal loss. Secondly, it was hypothesized that metabolic measures associated with IR may be important in carcinogenesis. It was shown that there are considerable diurnal variations and that a high fat diet (HFD) increased non-esterified fatty acids, triglycerides and insulin. This effect was more pronounced postprandially. Also, this HFD resulted in elevations in intracellular lipids of colon epithelial cells, spleen, muscle and liver. It was concluded that energy availability may be important in tissue defects associated with CRC and IR.
To my mom, a wonderful example of courage and faith
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LIST OF ABBREVIATIONS

AC      Aberrant crypt
AOM     Azoxymethane
ACF     Aberrant crypt foci
AUC     Area under the curve (OGTT curve)
BMI     Body mass index
CRC     Colorectal cancer
CHO     Carbohydrate
DAG     Diacylglycerol
DNA     Deoxyribonucleic acid
FA      Fatty acid
FAME    Fatty acid methyl ester
FFA     Free fatty acids
Ginf    Glucose infusion rate
HDL     High density lipoprotein
HF      High fat diet
IR      Insulin resistance
IRS     Insulin receptor substrate
LF      Low fat diet
MUFA    Monounsaturated fatty acids
NEFA    Non-esterified fatty acids
NIDDM   Non-insulin dependent diabetes mellitus
OGTT    Oral glucose tolerance test
PDH     Pyruvate dehydrogenase
PFK     Phosphofructokinase
PUFA    Polyunsaturated fatty acids
RR      Relative risk
SEM     Standard error mean
TG      Triglycerides
TNF     Tumor necrosis factor
VLDL    Very low density lipoprotein
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Chapter 1
CHAPTER 1: INTRODUCTION

Colorectal cancer (CRC) is associated with diets high in fat and energy and low in complex carbohydrates. McKeown-Eyssen and Giovannucci observed that these same diets have also been identified as risk factors for a condition known as insulin resistance, which is characterized by an impairment in insulin-mediated glucose uptake and increased serum levels of triglycerides (TG), insulin and glucose. These investigators hypothesized that alterations in these metabolic parameters seen in insulin resistance (TG, insulin and glucose) may be involved in tumor development. This idea was tested by examining the effect of diets high in fat, energy and n-3 fatty acids on markers of IR and CRC. Results showed these markers to be correlated with each other and with energy intake and weight gain. Together these results are consistent with the notion that energy availability may play a role in the manifestation of these conditions.

Several questions stemming from these observations are addressed in the work presented here, they are:

- Do other dietary factors influence measures of promotion, insulin resistance and energy availability in the same manner?

- Are alterations of insulin and energy substrates namely glucose, triglycerides and non-esterified fatty acids that are associated with insulin resistance important in carcinogenesis?
• Are the changes in these energy substrates reflected as alterations in the intracellular environment of colonic epithelial cells?

To answer these questions, this work is separated into two major projects.

Dietary calcium has also been observed to influence both promotion and insulin sensitivity, therefore an attractive agent to examine in light of the IR/CRC promotion hypothesis. Dietary calcium has been shown to reduce colon tumor promotion in several studies, (Baron et al., 1999, Pereira et al., 1994, Pence BC and Buddingh F, 1988, Wargovich MJ et al., 1990, Sitrin MD et al., 1991) but not all. (Kampman E et al., 1994). The manner in which calcium influences carcinogenesis has been primarily attributed to the complexing of bile acids and fatty acids, thereby reducing the availability of these bile acids to damage the colonic epithelium which may stimulate compensatory hyperproliferation. In addition to the controversy surrounding the efficacy of calcium supplementation as a preventative agent, there is a lack of a systemic hypothesis to explain observations of calcium’s protective effect on other cancer etiologies.

Alterations in metabolic parameters (insulin and triglycerides) associated with insulin resistance were also correlated with tumor promotion (Koohestani et al., 1998) suggesting that they may be important in carcinogenesis. Also, early results from a case-control study suggest that patients with cancer and with
colonic polyps have higher levels of circulating insulin and triglycerides than matched control patients (McKeown-Eyssen and the Toronto Polyp Prevention Group, 1996). Diurnal variations in these factors exist which need to be characterized in order to determine their potential importance in carcinogenesis. Diets which are high in fat have been attributed to contributing to elevations in circulating insulin, glucose, triglycerides and free fatty acids which are associated with the development of insulin resistance (Storlein et al., 1986). Insulin resistance has also been related to increases in intracellular lipid of the liver, muscle and adipose tissue which has been attributed to contributing to the clinical effects associated with insulin resistance (Storlein et al., 1991). In the same manner, diets that provide excess energy have been identified with an increase in colon cancer risk. The presence of potentially promoting intracellular lipid has not yet been investigated in colonic epithelial cells, which could provide insight into alterations at the cellular level in response to diet.

This thesis further explores the McKeown-Eyssen and Giovannucci hypothesis and the role that energy availability may play. Chapter 2 reviews the literature surrounding the role of calcium in carcinogenesis and insulin resistance and metabolic changes that occur in insulin resistance. Chapter 3 outlines the rationale for this work, hypotheses and objectives. Chapter 4 and 5 present the experiments conducted for this thesis. With the help of my supervisors Drs. Bruce and Sarma, I designed, conducted and analyzed these experiments, also
benefiting from the help of others (see Acknowledgements). Chapter 6 is a general discussion and suggestions for future work.
Chapter 2
CHAPTER 2: LITERATURE REVIEW

2.1 Colon Cancer

Colon cancer is the second leading cause of cancer mortality in the Western countries. In Canada, there is estimated 16 000+ new diagnosis of colorectal cancer and an expected mortality of 6300 patients - second only to lung cancer (Cancer Bureau, LCDC, Health Canada/NCIC). There are both genetic and non-genetic determinants that have been identified as contributors to the development of colon cancer. Although genetic predisposition plays an important role in cancer causation, environmental factors are believed to be responsible for up to 90% of colon cancer incidences (Doll and Peto 1981). Migration studies demonstrating an increase in colon cancer incidence rates in individuals among groups moving from low to high incidence areas support the notion that environmental factors are important (Haenszel and Kurihara, 1968). Moreover, the association of dietary patterns and colon cancer incidence suggests that diet is one of these important environmental factors. Early studies noted positive correlation's with total energy intake and with energy containing nutrients such as fat, protein and carbohydrates in case control and cohort studies with increases in colon cancer cases (Potter et al., 1993, Willet et al., 1990, Giovannucci 1992). Negative correlations have been demonstrated with diets that are high in vegetables, fruits, cereal and fiber, as well as lifestyles with exercise (Steinmetz and Potter, 1991).
2.2 Colon Cancer Development

Colon cancer development is currently described in three separate stages. The first is an initiation event where cells are mutated resulting in a loss or acquisition of genetic changes that can lead to abnormal control of cellular functions including proliferation and differentiation. The second stage is characterized by the promotion of the altered cells, which confers a proliferative advantage over the normal cells. The final stage is progression where the abnormal cells acquire characteristics that are seen in malignant tumors (Bruce et al. 1993).

During the promotion and progression stages of tumor development, the process in the colon has been described as the adenoma-carcinoma sequence. Here, an initiation or mutation event that alters an epithelial cell results in aberrant proliferation and the formation of a small polyp or adenoma. These adenomas are benign, but are believed to be precursors of carcinomas. Some of these adenomas will continue to grow, whilst acquiring and accumulating genetic alterations which are thought to contribute to the malignant phenotype, see figure 2.1. A model of these genetic alterations has been described, in which a sequence of genetic changes associated with each of these phenotypic stages has been identified (Fearon and Vogelstein, 1990, Vogelstein et al., 1988). The final stage of tumor development is the adeno-carcinoma where the adenoma has invaded and penetrated the underlying mucosa.
Figure 2.1. The multi-step process of colon carcinogenesis (Bruce et al., 1993).
Diet is thought to play a role at each of the stages of initiation, promotion and progression. For example, foods that contain chemicals such as heterocyclic amines or generate free radicals upon metabolism can initiate or cause mutations in DNA. After these genetic mutations and the subsequent replication of the DNA, mechanisms that normally repair the DNA are often unable to do so. Several dietary factors are thought to influence the proliferation or growth of genetically altered cells, these include diets that are high in fat. A reduction in cell turnover has been observed in calorie-restricted diets as well as diets high in fibre (Ames et al, 1995).

2.2.1 Aberrant crypt foci as biomarkers of colon cancer risk

Aberrant crypt foci (ACF) are putative precursor lesions of colon tumors that are thought to precede adenomas in the adenoma-carcinoma sequence of tumor development. The ACF are an early stage event characterized by an elongated opening, increase in crypt size, crowding of their nuclei and a thicker epithelial lining (McLellan and Bird, 1988). These ACF have been examined for molecular changes and dysplastic features that are associated with tumors (Roncucci et al., 1991, Stopera and Bird, 1992, Shivapurkar et al, 1997). As demonstrated, there is some evidence of dysplastic features associated with the ACF such as mutations in the k-ras gene, however it is not clear whether these ACF are preneoplastic (Davies and Rumsby, 1998). It has been suggested that
some of the ACF may regress and disappear where others may eventually lead to tumor formation.

After treatment with methylene blue, these ACF can be visualized under light microscopy. ACF have been used in many investigations as biomarkers for colon tumors since they were initially described.

2.3 Diet and Cancer

Diet has been suggested to account for up to 1/3 of cancer cases in the United States (Doll and Peto, 1981). Many dietary factors have been investigated since the early epidemiological observations. For example, diets that are calorie-restricted have been shown in animal models to have a significant reduction in both mitotic rates and tumor incidence. Consumption of fruits and vegetables has been consistently associated with a reduction in cancer incidence in many studies, and has been attributed in part to the presence of antioxidants. Diets that increase the risk of colon cancer formation include diets that are high in saturated fat, energy and low in fibre (Ames et al., 1995).

2.3.1 Dietary Fat and Colon Cancer

Both the amount and type of fat has been found to be important in tumor development in animal studies. Diets high in saturated and n-6 polyunsaturated fatty acids have been associated with increases in tumor incidence and ACF size
in carcinogen-initiated animals (Reddy et al., 1977, Reddy 1987, Reddy et al., 1991, Lafave et al., 1994). Fat is thought to act at the promotion stage of cancer progression. This is supported by studies demonstrating increased tumor incidence only after carcinogen administration in animal models on a high fat diet (Reddy et al., 1992). The lack of promotional effects on colon tumors by high levels of fats such as olive oil and marine oils suggested that fatty acid composition of dietary fat is important. Subsequent studies showed that fats containing n-3 fatty acids result in a reduction in tumor incidence as compared to control animals on a n-6 fatty acid containing corn oil diet (Reddy and Maruyama, 1986, Reddy and Sugie, 1988). The beneficial effects of n-3 fatty acids is supported by epidemiological observations where Greenland Eskimos who consume a large amount of fish in their diet have a lower prevalence of cancer rates than Western populations (Blot et al, 1975).

2.3.2 Calcium and CRC

2.3.2.1 Epidemiological Data supporting Calcium Supplementation

Dietary calcium supplementation has also been associated with effects on colon cancer incidence and development. Several epidemiological studies support the notion that calcium supplementation may have significant protective effects (Garland et al, 1985, Slattery et al, 1988). More recently, in a randomized controlled trial, patients with a recent history of colorectal adenomas receiving
dietary calcium supplementation were associated with a reduced risk of recurrent colorectal adenomas (Baron et al., 1999, Hofsted et al., 1998). In a prospective study, Hyman J et al. found an association between high dietary calcium intake and a reduction in the risk of recurrent adenomas, where the protective effect seemed to be greatest in individuals who also consumed a high fat diet (Hyman et al., 1998). Despite the support for the association between calcium and a reduction in cancer risk, there remains controversy. There are several studies that found no significant protective effect associated with calcium or calcium-containing products. One such cohort study found no relationship between total calcium intake and risk for adenomas (Kampman E et al., 1994). In two case-control studies comparing newly diagnosed adenomas with controls, and recurrent adenomas with controls found no association with calcium supplementation (Neugut et al., 1996).

2.3.2.2 Experimental Data supporting Calcium Supplementation

Several animal studies suggest that calcium supplementation is associated with a reduced risk for cancer development. Experimental models have demonstrated that reduced dietary calcium and vitamin D intake are associated with an increase in tumor promotion with more pronounced effects in animals on a high fat diet (Wargovich et al., 1990, 1996). In another study, animals which were on a Western-style diet consisting of reduced calcium and vitamin D levels and fat levels of the average human Western diet, had significant increases in proliferation of epithelial cells as compared to animals on
the control diet. Subsequent addition of dietary calcium and vitamin D markedly suppressed the hyperproliferation induced by the Western-style diet (Xue L et al., 1999).

Aberrant crypt foci, putative precursor lesions have been used as biomarkers for tumor formation. Several investigations have found a relationship between calcium supplementation and a reduction in the size or number of ACF in carcinogen-initiated animal models, suggesting that dietary calcium is protective against cancer growth. (Pereira et al., 1994, Pence and Buddingh, 1988, Wargovich et al., 1990, Sitrin et al., 1991). For example, in animals fed diets containing between 5 – 15 mg calcium/kg diet, where 5.0 mg/kg diet is the AIN76 standard level, there was a decrease in total tumor incidence (p=0.12) and significant decrease in dysplastic tumors (Beaty et al., 1992, Belbraouet S et al., 1996). In diets ranging from 2 – 20 mg calcium/kg diet, there was a significant alteration in the incidence of adenomatous polyps. Instead of an inverse relationship between calcium and ACF size, Li et al. observed a multiphasic dose-response curve where there were smaller ACF at both low and high concentrations as compared to levels normally found in the standard animal diet (Li et al., 1998). Consistent with these observations, a similar relationship has been found with polyp formation in response to dietary calcium (Karkare et al., 1991).
2.3.2.3 Relative efficacy of different calcium salts

Although there is general agreement of the role of calcium as a protective agent in colon cancer promotion, there lies some controversy in the relative efficacy of the counterion that should be used for calcium supplements. Pereira et al., considered the effects of different calcium salts on AOM-induced ACF in the rat colon. They demonstrated that the calcium salts of carbonate, glucarate and chloride inhibited ACF growth, whereas lactate and phosphate did not (Pereira et al., 1994). Contrary to these observations, Belbraouet et al. showed that calcium lactate and gluconate were superior at preventing the formation of carcinogen-induced tumors than calcium carbonate.(Belbraouet et al., 1996). Colonic cell proliferation was reduced to a greater extent by calcium phosphate as compared to calcium casein or lactate (Lupton et al., 1995) Together these studies highlight the disagreement in the literature as to the most effective form of calcium supplementation.

2.3.2.4 Calcium, Phosphate and Vitamin D Homeostasis

The importance of the form that calcium is delivered for colorectal tumor promotion is due in part to the complex regulatory system which is in place to balance calcium and phosphate ions which is also known to involve vitamin D. As reviewed by many, changes in serum calcium concentrations is influenced by the actions of parathyroid hormone (PTH) and the active metabolite of vitamin D,
namely 1,25 dihydroxycalciferol (1,25(OH)₂D), both which act to reabsorb and mobilize calcium from the kidneys and bone, respectively. For example, in hypocalcemia there is an increase in the mobilization of calcium and phosphorous through the increased participation of PTH and 1,25(OH)₂D, this allows for calcium mobilization and a return to steady state conditions. As the concentrations of serum calcium fluctuate, so do levels of phosphorous. The intestinal absorption of both calcium and phosphate ions is dependent on dietary intake and stimulate by 1,25(OH)₂D. The regulation of both ions is also influenced by the other’s absorption where each can act to limit the absorption of the other. In light of the work to be presented in this thesis, two issues are of importance. The first is that 1,25(OH)₂D concentrations vary in order to maintain both serum calcium and phosphate ion concentrations, and second that each ion can influence the others absorption. This implies that in order to consider the effects of 1,25(OH)₂D and calcium on colon cancer promotion, keeping the ratio of calcium to phosphate ions may be important.

2.3.3 Vitamin D and CRC

Observations of a relationship between sunlight exposure and colon cancer incidence rates initiated investigations of the role of vitamin D metabolites and colorectal cancer (Garland and Garland, 1980). Both epidemiological and experimental work have demonstrated that vitamin D may protect against colon cancer growth. (Martinez and Willet, 1998). In vitro studies have demonstrated that the vitamin D metabolite, 1,25 dihydroxycalciferol (1,25(OH)₂D), inhibits
proliferation of human colorectal cells, colorectal cancer and adenoma cell lines (Lointier et al., 1987, Thomas et al., 1992, Shabahang et al., 1994). The receptors for 1,25(OH)_{2}D which are believed to be required for the anti-mitogenic activity of vitamin D, have been detected in normal-appearing colonic epithelium as well as adenocarcinomas (Lointier et al., 1987, Meggouh et al., 1991). These same receptors have also been identified in colon cancer cell lines and well-differentiated tumors (Frampton et al., 1982, Cross et al., 1996), suggesting that a manner in which 1,25(OH)_{2}D can enter and potentially influence cells.

As with dietary calcium, there are some inconsistencies in the literature of the effect of 1,25(OH)_{2}D on colon cancer promotion. Some studies have demonstrated a reduction in tumor incidence (Pence and Buddingh, 1988) while others have found no effect on tumor incidence (Comer et al., 1993) nor on ACF growth (Wargovich et al., 1996).

2.3.4 Possible Mechanisms of how Diet Influences Cancer Development

Traditionally, dietary fat is thought to influence colon tumor development through the increased production of bile acids (Cummings et al., 1978). Fat increases the concentration of secondary bile acids namely deoxycholic acid and lithocholic acid which have been shown to be tumor promoting. This is supported by epidemiological studies which have shown an increase in biliary steroids in the feces of populations at high risk (Americans) as compared to those of a Japanese population that is at lower risk. Activation of protein kinase C by bile
acids in colonic epithelium may represent a mechanism by which bile acids influence intracellular signaling events. (DeRubertis et al., 1984).

These bile acids may also be altered or modified by the microflora of the colon. A structural similarity between steroids such as bile acids and polycyclic aromatic carcinogens has been demonstrated in early studies, suggesting that the conversion from naturally occurring bile acids to even more harmful metabolites is chemically feasible and may take place in vivo. Moreover, several studies have found higher levels of fecal steroids in high incidence populations and differences in the intestinal bacteria population, where higher incidence areas had bacteria that can metabolize steroids more readily. (Aries et al., 1969, Hill et al., 1971).

An alternate mechanism by which dietary fat may effect proliferation rates is by the autoxidation of polyunsaturated fatty acids to hydroxy or hydroperoxy fatty acids. Bull et al. demonstrated that initial oxidation products of linoleic acid stimulates DNA synthesis in the colon of an animal model (Bull et al., 1984).

Newmark et al., postulated a mechanism by which calcium may interact with the damaging fatty acids and bile acids produced in response to a high fat diet. They suggested that the toxic bile acids would form insoluble soaps with ionized calcium, rendering them inert and non-damaging. The calcium-bile acid
complex can then be excreted without harm to the epithelium. (Newmark et al. 1984)

As highlighted, many of the mechanisms postulated to explain the relationship between diet and colon cancer are based on factors such as bile acids, which interact with the colonic epithelial cells from the luminal surface. However, fewer mechanisms explain diet’s effect on the colon via the systemic circulation.

A systemic effect of diet on tumor development is exemplified by the role of dietary calcium. Calcium has been shown to influence tumor development in other cancers, such as cancer of breast supported by epidemiological observations of a relationship between intake of dairy products, specifically milk and a reduced risk of breast cancer (Knekt P et al., 1996, Jarvinen R et al., 1997). Proliferation at the mammary terminal ducts and expansion of the proliferative epithelial cell compartment was also found in animals on a Western-style diet containing reduced levels of calcium. (Zhang L et al., 1987, Khan N. et al., 1994). These observations cannot be explained by toxic effects of bile acids directly on epithelial cells. This infers that a broader based mechanism should be developed in order to, (1) explain the effect of other dietary factors currently unaccounted for by mechanisms at the luminal surface and, (2) to account for observations of the influence of diet on other cancer etiologies.
2.4 McKeown-Eyssen and Giovannucci Hypothesis

McKeown-Eyssen and Giovannucci noted the striking similarity between lifestyle risk factors for colorectal cancer and insulin resistance, the latter that may precede non-insulin dependent diabetes mellitus (NIDDM). These include diets that are high in fat and energy and low in complex carbohydrates, as well as lifestyles that are sedentary. They hypothesised that these lifestyle factors may contribute to the development of insulin resistance, a condition which is associated with elevated levels of insulin, glucose and triglycerides, and that insulin acts as a tumor promoter. McKeown-Eyssen went further to suggest that the elevated levels of energy provided by glucose and triglycerides might also be involved in promoting the growth of neoplastic cells (McKeown-Eyssen 1994, Giovannucci 1995).

2.4.1 Evidence to support this hypothesis

Several epidemiological have shown an association between NIDDM and colon cancer development. As reviewed by Kim, several case-control and prospective studies have shown an association between patients with diabetes and an increased risk of developing colon cancer. These studies were limited due to the smaller sample sizes, small number of cases of colorectal cancer, and often failure to control for confounding variables. Two recently published prospective studies provide more evidence of a positive association between
diabetes and colon cancer. The first was a cohort study with over 150,000 patients with diabetes mellitus, in these patients there was an average 39% increased risk in developing colon cancer (Weiderpass et al., 1997). The second study with over 1 million subjects found that men with diabetes had a significant 30% increase in developing colon cancer whereas women had a non-significant 16% increase in risk (Will et al., 1998). Unlike the aforementioned study, this investigation adjusted for colorectal cancer risk factors including age, race, socioeconomic status, smoking, alcohol, dietary intake of aspirin use, physical activity and family history of colon cancer (Kim, 1998).

Early results by a case-control study suggest that patients with cancer and with colonic polyps have higher levels of circulating insulin and triglycerides, factors that are associated with insulin resistance, than matched control patients (McKeown-Eyssen and the Toronto Polyp Prevention Group, 1996).

The relationship between insulin resistance and colon cancer has been tested directly in animal models where exogenous insulin was injected daily into rats after initiation with a colon carcinogen. Exogenous insulin was demonstrated to promote the development of colonic tumors and aberrant crypt foci, putative precursor lesions (Tran et al., 1996, Corpet et al., 1997). The effect of a high fat diet on markers of both insulin resistance and tumor promotion was assessed in an initiated animal model. This study found that animals on the high risk, high fat
diet had demonstrated a decrease in insulin sensitivity and an increase in ACF promotion (Koohestani et al., 1997).

2.5 Insulin Resistance

Insulin resistance is a condition that is characterized by an impairment in insulin-mediated glucose uptake in peripheral tissues. There is an increase in fasting insulin and VLDL triglycerides. Both genetic and non-genetic factors influence the development of a reduced sensitivity of insulin stimulation on peripheral cells, leading to impaired glucose uptake. The inability for these resistant cells to uptake glucose stimulates a compensatory increase in the secretion of insulin, reflected as elevated levels of insulin and glucose in the circulation. Among the non-genetic contributors to the development of insulin resistance are diet, obesity and low physical activity.

2.5.1 Pathogenesis of Insulin Resistance

There are a number of different mechanisms that have been described and refined since the inception of the concept of insulin resistance; they can be broadly divided into three different categories. The first is the notion that antagonists of both the antibody and enzyme class are directed at the insulin molecule itself not allowing for its interaction at the surface of target cells. Although this area has been studied in detail, it is now known that these
mechanisms only account for a small fraction of insulin resistance in the human population. The second group of mechanisms involve genetic or primary defects in the ability of insulin to signal its target cells. This includes genetic mutations in the major substrate of the insulin receptor, IRS-1 and other pathways whose ability for phosphorylation/dephosphorylation have been altered. The third group of possible mechanisms includes local and circulating factors that may impinge on target cells, bringing about insulin resistance. These factors could be as a result of genetic mutations or molecules such as glucose, free fatty acids or cytokines such as TNF-α (Hotamisligil et al., 1993).

2.5.2 Role of insulin resistance in the development of NIDDM

The precise role of insulin resistance in the development of NIDDM is not known. There are two main hypotheses that explain the role of insulin resistance in the production or exacerbation of NIDDM, see figure 2.2. The first is based on observations from several prospective studies which have shown that hyperinsulinemia predicts the onset of NIDDM. This led investigators to postulate that insulin resistance due to genetic and/or environmental factors leads to a necessary increase in insulin secretion by the pancreas. It is hypothesized that in some individuals the pancreas becomes “exhausted” also known as β-cell fatigue, resulting in a reduction in insulin secretion leading to an increase in plasma glucose. The second hypothesis suggests that the defect in insulin secretion is one of the earliest events in the development of NIDDM. There is a reduction in circulating insulin that leads to an increase in plasma glucose. It is
Figure 2.2: Insulin Resistance and the development of NIDDM

**HYPOTHESIS 1:**
Insulin resistance as the initiating event in NIDDM

- Insulin resistance due to genetic and/or environmental factors → ↑ insulin secretion by the pancreas → hyperinsulinemia → Impairment in insulin action → Pancreas unable to increase insulin further (due to genetic/environmental factors?) → β-cell fatigue, decline in insulin secretion and increase in plasma glucose → NIDDM

**HYPOTHESIS 2:**
Defect in insulin secretion as the earliest event in NIDDM

- Defect in insulin secretion → Insulin deficiency leads to hyperglycemia → Insulin resistance and secondary hyperinsulinemia → Hyperglycemia
this hyperglycemia which is postulated to lead to insulin resistance and secondary hyperinsulinemia. Despite the differences in the chronological order of events leading to the development of NIDDM, it is agreed that there is both an impairment in insulin action and secretion preceding the onset of NIDDM (Flier 1993).

2.5.3 Measures of Insulin Resistance in vivo

There are several measures for insulin resistance in vivo, these include the hyperinsulinemic-euglycemic clamp, glucose and insulin tolerance tests, fasting and postprandial levels of insulin, glucose and triglycerides, see table 2.1. The oral glucose tolerance test is used as an indirect measure of insulin resistance. Here, a bolus of glucose is given orally after a period of fasting. Blood glucose and/or insulin are measured in the fasting state and after the glucose challenge. An elevated and prolonged level of insulin and glucose over the time period after the glucose challenge implies insulin resistance. Area under the curve of the glucose and/or insulin concentrations quantifies this measure of insulin resistance. The euglycemic-hyperinsulinemic clamp gives a continuous infusion of insulin and then infuses glucose at a rate that maintains or "clamps" the blood glucose at a predetermined concentration (normal range being euglycemia). The rate of glucose infusion reflects the efficiency of the peripheral cells to remove the glucose from the circulation. As the degree of insulin resistance increases,
Table 2.1: Selected Methods used in assessment of insulin sensitivity *in vivo*

<table>
<thead>
<tr>
<th>METHOD</th>
<th>DESCRIPTION</th>
<th>ADVANTAGES/DISADVANTAGES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Glucose Tolerance Test (OGTT)</td>
<td>• A standard glucose load is administered, after which plasma insulin and/or glucose are measured. The glucose/insulin ratio is calculated to give an indication of the degree of insulin resistance.</td>
<td>• Poorly reproducible, variation in glucose absorption</td>
</tr>
<tr>
<td>Yalow and Berson, 1960</td>
<td></td>
<td>• G/I ratio is dependent on both insulin clearance as well as secretion</td>
</tr>
<tr>
<td>Minimal Model</td>
<td>• I.V. bolus of glucose is given and plasma glucose and insulin are sampled frequently over 180 minutes following bolus.</td>
<td>• Gives index of insulin action and secretion</td>
</tr>
<tr>
<td>Bergman et al, 1979</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homeostasis model assessment</td>
<td>• Basal (fasting) plasma glucose and insulin</td>
<td>• Simple, no artificial stimulus involved</td>
</tr>
<tr>
<td>Mathews and Turner, 1979</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperinsulinemic-Euglycemic clamp</td>
<td>• Insulin is infused systemically at a continuous rate, plasma glucose is maintained by constant glucose infusion. Glucose is “clamped” at either physiological/ambient (euglycemic) or above (hyperglycemic). Glucose infusion rate ($G_{inf}$) equals the sum of the decrease in hepatic glucose release and the increase in glucose uptake.</td>
<td>• Invasive, dependent on skill of investigator.</td>
</tr>
<tr>
<td>DeFronzo et al., 1979</td>
<td></td>
<td>• Can generate dose-response curves without interference of counterregulatory hormones.</td>
</tr>
</tbody>
</table>

\[
G_{inf} = \downarrow \text{hepatic glucose release} + \uparrow \text{glucose uptake}
\]
less glucose is required, as the cells are less able to remove the glucose from the blood. Since the liver takes up very little glucose during euglycemia, an increase in glucose uptake can be attributed to enhanced disposal by extra-hepatic tissue. These measures differ in the sensitivity of the detection and determination of the degree of insulin resistance. The euglycemic-hyperinsulinemic clamp is the direct and standard method, but requires invasive catheterization. The oral glucose tolerance test is less invasive and provides a reasonably sensitive and precise measure of insulin resistance (Bergman et al., 1985).

2.6 Diet and Insulin Resistance

2.6.1 Dietary Fat and Insulin Resistance

Using the hyperinsulinemic-euglycemic clamp, high fat feeding has been found to impair insulin stimulated glucose uptake in both adipose and muscle tissue in experimental animal models (Storlein et al., 1986). When insulin action is assessed in individual tissues, studies demonstrate insulin resistance occurring first in the liver followed by more widespread resistance of muscle and adipose tissue. Associated with the high fat diet was an accumulation of storage lipid in muscle (which is consistent with the Randle glucose-fatty acid cycle where an increase in lipid contributes to impairment in glucose utilization)(Randle et al., 1963). Interestingly, as with tumor promotion the type of fat plays an
important role in insulin action. Animal studies have demonstrated that inclusion of n-3 fatty acids in the high fat diet prevented insulin resistance in both the liver and muscle (Storlein et al., 1987). The fatty acid profile of the diet may influence insulin action through alterations in membrane lipids. In fact, several studies have shown a correlation between the percentage of long chain polyunsaturated fatty acids in the phospholipids of muscle tissue and insulin sensitivity. The relationship between amount and type of fat on insulin action in animal models is also supported in human studies and epidemiological investigations. (Storlein et al., 1996).

2.6.2 Dietary Calcium and Insulin Resistance

Supplemental dietary calcium intake has been shown to be associated with a reduced risk of the development of non-insulin dependent diabetes mellitus. In the Nurses Health Study, a large prospective analysis of several dietary factors was conducted to determine associations with increases risk in the development of NIDDM in women. After controlling for body mass index (BMI), family history of developing diabetes, weight change over the test interval and alcohol intake, there was a significant inverse relationship between calcium intake and the risk of developing diabetes (RR=0.85, 0.53-1.37). This trend was in lean subjects (BMM< 29) and to a lesser extent in obese women (Colditz GA et al., 1992). Calcium supplementation has also been shown into influence insulin sensitivity in hypertensive patients. The improvement in insulin sensitivity is
attributed to a decrease in intracellular calcium seen in platelets from these patients (Sanchez et al. 1997).

2.7 Pathogenesis of NIDDM and effects on the metabolic milieu and physiological alterations in tissue

As suggested by the different measures of insulin resistance in vivo, levels of circulating insulin, glucose, fatty acids and triglycerides can be used as indicators of systemic insulin resistance. The fluctuations in these parameters reflect a complex system of physiological changes. Some of the underlying mechanisms behind these variations will be discussed here.

2.7.1 Glucose-fatty acid cycle

The relationship between energy substrates namely glucose and fatty acids, was suggested to act in a competitive manner by Randle and colleagues some 30 years ago. Here they hypothesized that elevations in circulating free fatty acids can influence glucose metabolism at several steps in the metabolic pathway. As seen in figure 2.3, there appears to be an inverse relationship between the metabolism of glucose and fatty acids. When there is an increase in circulating free fatty acids there is a down-regulation of the glucose metabolic pathway through the inhibition of key enzymes including phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH). There is also a selective depletion and elevation in oxidizing and reducing equivalents respectively, needed for
Figure 2.3: Glucose-fatty acid cycle

An increase in fatty acids (FFA) results in a reduction of glucose metabolism through inhibition of key enzymes, phosphofructokinase (PFK) and pyruvate dehydrogenase (PDH). There is also a decrease in oxidizing and reducing agents required for glucose utilization, (NAD/NADH)

Based on this relationship between glucose and free fatty acids, combined with several in vivo investigations, it has been suggested that elevations in circulating free fatty acids causes a decrease in glucose disposal and increase in hepatic glucose release in NIDDM. However, although alterations in fat metabolism are thought to play a role in NIDDM the extent to which this contributes to the development of NIDDM remains to be defined. In spite of this inverse relationship between glucose and free fatty acids, this does not necessitate causality. This is highlighted in observations where non-obese individuals with NIDDM have normal lipid oxidation, yet have impairment in glucose metabolism (Golay et al. 1988).

2.8 Sites of Insulin Resistance (hepatic vs. peripheral)

2.8.1 Fasting Response

Circulating levels of glucose, free fatty acids and insulin are a reflection of a complex and tightly regulated system balancing energy substrates and their metabolic pathways. In normal individuals, a bolus of glucose results in the stimulation of the pancreas to secrete insulin. This increase in glucose and insulin leads to enhanced glucose uptake by peripheral insulin-sensitive tissues,
primarily the muscle. The rising insulin and glucose suppresses hepatic glucose production, which together maintain euglycemia. In individuals with NIDDM, pancreatic β-cell insulin secretion is impaired, hepatic glucose production is increased and peripheral insulin-stimulated glucose uptake is diminished.

Hepatic glucose output has been quantified in several studies and found to be consistently elevated in patients with NIDDM (Revers et al., 1984, Dineen et al., 1992). Interestingly although both obese and non-obese individuals with NIDDM demonstrate increases in hepatic glucose production, individuals with impaired glucose tolerance show no increase in hepatic glucose output as compared to normal controls (Kolterman et al., 1981, Olefsky 1989). In subjects with NIDDM, a close correlation between fasting serum glucose and hepatic glucose output has also been demonstrated, suggesting that the fasting hyperglycemia seen in NIDDM is a result of the increase in hepatic glucose production. (Kolterman OG et al., 1981, Bowen et al., 1973). This notion is also supported by the location of glucose disposal in the basal or fasting state. Insulin-dependent glucose uptake only accounts for approximately 20-30% of the glucose disposal, where the majority of glucose during the fasting state is being taken up by the central nervous system. The cause of this increase in hepatic glucose output may be attributed to a variety of factors including hepatic insulin resistance and an increase in gluconeogenic substrate availability (Baron et al., 1987, Efendic et al., 1985). Elevations in free fatty acids have also been suggested as a cause for an increase in hepatic glucose output, where
elevations in fatty acid oxidation are thought to provide the energy required for gluconeogenesis seen in NIDDM (Groop et al., 1991).

Diabetic dyslipidemia is characterized by elevations in fasting triglycerides, especially VLDL triglyceride and a reduction in HDL cholesterol. There is enrichment of these lipid particles with triglyceride, which in the presence of hyperglycemia may further impair the removal of these triglyceride-rich particles. The elevation in insulin also contributes to the lack of a compensatory increase in lipoprotein lipase activity that acts to break down lipids and allow for uptake into the cell. The combination of increased production and a decrease in the clearance of these triglyceride-rich particles contributes to circulating hypertriglyceridemia. (Kreisberg 1998).

2.8.2 Postprandial response

In the postprandial state, approximately 40% of the circulating glucose is taken up by the liver, where the remaining 60% is primarily taken up by the muscle. This infers that postprandial glucose concentrations are a reflection of both hepatic and peripheral tissue glucose utilization.

Dyslipidemia seen in the fasting states is exacerbated postprandially as there is even more triglyceride available from the diet. This is demonstrated by an interesting correlation between fasting triglyceride levels and area under the
curve for triglycerides after a high fat meal suggested by Lewis et al. Here, an increase in fasting triglyceride levels within the normal range corresponded to a much larger increase in area under the curve for serum triglyceride concentrations after high fat feeding. This suggests that patients with only a modest increase in fasting triglyceride levels may have much higher postprandial triglyceridemia. This suggests that there is much higher systemic exposure to triglycerides than what is represented by fasting values. In fact, only 30-40% of diabetic patients have triglyceride levels above 200 mg/dL and only 10% exceed 400 mg/dL, but alterations in HDL and LDL particles are apparent in all these patients. (Lewis et al, 1991, Kreisberg, 1998).

It has been suggested that there may be abnormalities in the entrapment of lipoprotein lipase-derived fatty acids in the postprandial state allowing for some fatty acids to enter into the circulation. As suggested by the glucose-fatty acid cycle put forth by Randle and colleagues, elevations in fatty acids impair glucose uptake. Since the rate of hepatic VLDL-TG production is dependent on the supply of fatty acids, an increase in VLDL-TG is expected contributing to hypertriglyceridemia. (Randle et al., 1963, Frayn 1998).
2.8.3 Experimental Evidence supporting Alterations in the Metabolic Milieu in Response to Diet

As reported here, several studies have considered the effect of a high fat diet on insulin levels. Many, but not all studies report an elevation in insulin in response to high fat feeding. Insulin has diurnal pancreatic secretion with levels changing in response to diet and activity. Iwashita et al. found no change in insulin and glucose concentrations over a 24-hour period in animals fed a high fat diet for 4, 10 and 21 weeks. This is consistent with a study done by De Gasquet et al. who also showed no difference in 24-hour profiles of glucose and insulin between animals on a high fat diet and low fat controls. However, they did report significant mean elevations in plasma fatty acids and triglycerides. Interestingly, this difference was most apparent postprandially (Iwashita et al., De Gasquet et al., 1977).

2.8.4 Intracellular Triglycerides in Insulin Resistance

Elevations in tissue triglycerides have been described in both patients and animals models with NIDDM. The latter include models with diet and genetically-induced insulin resistance. High fat feeding is associated with increases in muscle tissue triglyceride levels. It is this increase in triglyceride availability which may contribute to the muscle insulin resistance (Kraegen et al., 1991). This elevation in triglyceride is also seen in liver tissue with animals fed a high fat diet.
(Yaqoob et al., 1995). Animal models with genetically initiated obesity also demonstrate significant increases in muscle, liver and pancreatic triglyceride levels (Koyama et al., 1997).

The type of fatty acids that make up the high fat diet can influence both insulin sensitivity and total triglyceride accumulation in muscle. Animals fed a diet containing n-3 fatty acids demonstrated an increase in insulin action as measured by the hyperinsulinemic-euglycemic clamp. There were no significant differences in basal or clamp levels of blood insulin, glucose, triglyceride or fatty acids. However, in muscle tissue there was a significant decrease in triglyceride levels. This group went further to demonstrate a strong negative correlation between fasting muscle triglycerides and insulin-stimulated glucose metabolism. This suggests that with an increase in stored triglyceride, there is a decrease in insulin action and that n-3 fatty acids increase insulin action and decrease muscle triglycerides. (Storlein et al., 1991). In addition to a reduction in the total amount of triglycerides, the fatty acid profile of the diet can influence the fatty acid composition of the triglyceride fraction of skeletal muscle. (Matsui et al., 1997)

The link between insulin resistance and tissue lipid availability and metabolism may be through alterations in kinases downstream of the insulin receptor. Aberrations in the protein kinase C isoenzymes have been shown to lead to decreases in insulin signaling and therefore an attractive candidate to
consider in light of increased lipid availability. In an experiment conducted by Schmitz-Peiffer et al., animals on the high fat diet were associated with a decrease in insulin sensitivity and an increase in muscle triglyceride and diacylglycerol. High fat feeding was also associated with changes in the expression and cellular localization of protein kinase C isoenzymes. These kinases are associated with insulin resistance, suggesting a mechanistic link between muscle lipid availability and insulin resistance (Schmitz-Peiffer et al., 1997)
CHAPTER 3: INTRODUCTION TO EXPERIMENTAL WORK

3.1 Rationale

McKeown-Eyssen and Giovannucci noted a striking similarity between risk factors for insulin resistance and colorectal cancer. They hypothesized that high risk diets lead to changes associated with insulin resistance and that these changes may contribute to cancer promotion. More specifically, they suggested that the underlying factor(s) may be elevations in insulin concentrations which can act as a tumor promoting agent and/or increases in energy substrates, namely triglycerides, NEFA and glucose, which can provide energy for cellular proliferation. The delineation as to whether one or both of these groups of circulating agents may be more important in colon cancer promotion was demonstrated in part by Koohestani et al. They showed a correlation between markers for both insulin resistance and ACF promotion across diets high in fat, energy and n-3 fatty acids. They also demonstrated that energy intake and weight gain were most closely correlated to ACF promotion, suggesting that energy availability may be important (Koohestani et al., 1998). This thesis tests whether the relationship between insulin resistance and tumor development can be attributed to alterations in energy availability. This is achieved by considering the role of dietary calcium, another dietary factor related to cancer growth and possibly NIDDM, on metabolic measures often associated with insulin resistance and ACF promotion in the same animal model. Controversies surrounding the protective effects of supplemental calcium are also elucidated using this
experimental system. In addition, this work seeks to characterize metabolic changes that occur as a result of a high risk, high fat diet and whether these said alterations are reflected inside the colonic epithelial cells thereby exploring further the potential role of energy substrates in carcinogenesis.

3.2 Hypothesis

Following from the McKeown-Eyssen and Giovannucci hypothesis relating insulin resistance and colorectal carcinogenesis, several questions can be asked. They are:

i. Is dietary calcium an agent that affects colon carcinogenesis through changes in metabolic measures?

ii. Are elevations in circulating growth factors ie. insulin and/or energy substrates important in the development of colon tumors?

iii. What is the nature these metabolic measures normally associated with insulin resistance, and are these alterations reflected inside the colon cell as excess energy potentially contributing to carcinogenesis?
3.3 Objectives

The objectives of this work are:

i. To determine the effect of calcium and vitamin D on aberrant crypt promotion.

ii. To determine whether supplemental calcium and vitamin D reduces and circulating energy substrates and indicators of insulin resistance and aberrant crypt promotion to the same extent.

iii. To delineate diurnal variations in circulating triglycerides, fatty acids, glucose and insulin in response to a high fat diet

iv. To see if diurnal changes in these circulating energy substrates are reflected inside the cell as alterations in amount triglycerides and fatty acids.
Chapter 4
4.1 Introduction

Colon cancer is the second leading cause of cancer death in the Western countries and is influenced by both genetic and non-genetic determinants. The environmental factors are thought to contribute up to 90% of the cases of colon cancer and include a sedentary lifestyle and diets that are high in fat and energy and low in complex carbohydrates (Doll and Peto, 1981). Dietary calcium has also been shown to influence tumor development. Several studies both epidemiological and experimental have demonstrated a protective effect of dietary calcium (Garland et al, 1985; Slattery et al, 1988; Hofsted et al., 1998; Baron et al, 1999), though the effect is not consistent across all studies (Kampman et al., 1994, Cats A et al., 1995, Neugut et al., 1996). The mechanism(s) by which calcium affects tumor promotion, however, is not clear.

The usual explanation for the protective effect of calcium is that products of fat digestion, particularly the secondary bile acids, are toxic to the colonic epithelium, either through exposure to bile acids in the fecal stream or, perhaps, to circulating bile acids (Cohen et al,1980; Galloway DJ et al, 1986; Bayerdoreffer E et al, 1994, 1995). Increased dietary calcium, by forming complexes with the bile acids, reduces the exposure of the colonic epithelial cells
to their toxic effects and consequentially reduces compensatory proliferation and promotion (Newmark et al., 1984). A problem with this hypothesis is that it does not explain the protective effect of calcium in the promotion of cancers at other sites (Carroll et al, 1991; Knect et al., 1996, Jarvinen et al., 1997) nor the failure of some studies to show that dietary calcium reduces fecal bile acids in fecal water (Alder, 1993).

A second explanation is possible with a recent hypothesis based on the observation that diets that lead to colonic cancer promotion are also diets that lead to increased circulating triglycerides, insulin resistance, insulin and glucose intolerance (McKeown-Eyssen, 1996, Giovannucci 1997). McKeown-Eyssen and Giovannucci suggest that high energy diets result in high levels of circulating insulin and/or energy substrates which then act as growth factors to promote the growth of initiated colonic cells and cancer precursor lesions. If their suggestion is correct, we might expect that high levels of dietary calcium would reduce promotion since it is known that calcium can reduce the digestibility of fat and increase fecal fat and fecal weight (Behling et al 1990; Appleton et al, 1991, Lupton et al, 1994). High levels of dietary calcium would therefore be expected to reduce recovered energy intake, to reduce insulin resistance and to reduce colon cancer promotion.

To test this expectation the effect of various levels of dietary calcium in low and high fat diets on the growth and number of aberrant crypt foci (ACF),
presumed precursors of colon cancer (Bird 1987, Bruce et al., 1993, Wargovich et al., 1996) was examined. At the same time the effect of the levels of dietary calcium on appropriate metabolic measures was assessed. The question to be addressed was, does calcium reduce colon cancer promotion at the same level that it reduces energy intake? Calcium was provided as calcium dibasic phosphate to reduce the possible effects of changes in calcium-phosphate ratios and the possible effects of vitamin D were also assessed.

4.2 Material and Methods

4.2.1 Animals

Male Fischer 344 rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing approximately 180 g were housed individually in wire-bottom cages. The temperature and humidity were controlled at approximately 22°C and 50%, respectively. The room was maintained on 12-h dark/light cycles, with the dark cycle extending from 7 p.m. until 7 am. Tap water from an automatic system was provided ad libitum. Care of the animals conformed to the guidelines of the Canadian Council on Animals Care, and the University of Toronto Animal Care Committee approved the protocol.
4.2.2 **Experimental Design and Diets**

The protocol scheme is summarized in figure 4.1. In both experiments, animals were acclimatized for approximately 1 week during which time they consumed Rodent Chow (Ralston Purina International, Strathroy, Canada). After acclimatization rats were initiated with the colon carcinogen azoxymethane (AOM) (Sigma, St. Louis, MO) at a dose of 20 mg/kg body weight. In experiment 1, 126 animals were randomized by weight into eight dietary groups one week later, where diets differed in levels of calcium, fat and vitamin D. In experiment 2, 118 animals were randomized into diets differing in levels of calcium and fat. In both experiments, an oral glucose tolerance test (OGTT) was performed at approximately day 69. Animal weights were measured bi-weekly and food intake at 29, 50 and 81 days after the diet began. At approximately day 100 animals were sacrificed at 9 p.m. and colons were removed and assayed for aberrant crypt foci (ACF).

Diet compositions for experiment 1 and 2 are described in Figure 4.1 caption (and in the Table 1 and 2 in the Appendix A). The diets were formulated by Dyets (Bethlehem, PA) and were based on the standard AIN76C diet (Bieri et al., 1977). The high fat diets were balanced on an energy density basis. Calcium levels ranged from 0.5 to 15.0 g/kg diet and was provided as calcium dibasic phosphate.
**Figure 4.1.** Protocol scheme for Experiments 1 and 2. Diets 1.8 and 2.7 are low fat (LF) control diets based on AIN-76A with 50 g/kg corn oil, 1g/kg vitamin D3 (1000 u/g) and 5g/kg calcium as calcium dibasic phosphate. Diets 1.4 and 2.3 are high fat (HF) diets based on AIN-76A with 300g/kg beef tallow and other components of the diet adjusted to the same concentration as AIN-76A on a caloric density basis, with adjustments made to the sucrose. Calcium is at the concentration indicated. High vitamin D (HD) is at the the concentration in AIN-76A; low vitamin D (LD) is at a 10 fold reduced level.
4.2.3 **Metabolic Measures**

For the OGTT, the animals were fasted for 7 h (7:00 am to 2:00 p.m.) and were then given an oral glucose gavage of 4 mg anhydrous glucose/g body weight. Blood glucose was measured from the tail vein using a glucometer (Medisense Canada, Toronto, Canada) prior to and 30, 60, 90 and 120 min after gavage. The total area under the glucose tolerance curve (a.u.c.) was calculated from 0 to 120 minutes and quantified using the trapezoid rule (Wolever et al., 1991). Blood samples were taken by cardiac puncture upon sacrifice. A glucose analyzer measured glucose concentrations. Triglycerides and free fatty acids were measured by colorimetric kits (kit 337 Sigma, Kit 994-75409 Wako, Richmond, VA). Insulin was measured using a radioimmunoassay (Rat insulin RIA kit; Linco Research, St. Charles, MO). In experiment 2, feces were collected over a 24 h period prior to sacrifice and their weights determined.

4.2.4 **ACF Promotion Measures**

ACF were assayed as previously described (Bruce et al, 1993). Briefly, colons were removed and rinsed with Krebs-Ringer bicarbonate buffer. They were cut longitudinally and fixed flat between pieces of filter paper in 10% buffered formalin. The colons were stained with approximately 0.2% methylene blue and examined under a light microscope at 40X magnification for ACF. The number of ACF per colon and the number of crypts comprising each foci were
recorded (Bruce et al., 1993).

4.2.5 **Statistical Methods**

The students t-test using unequal variance and the Bonferroni test of multiple comparisons were used to determine statistical significance between groups after establishing significance with one and two-way ANOVA's. In experiment 1 the interactions between the effects of dietary levels of calcium, fat and vitamin D on the measured variables were assessed using a three-way ANOVA. Statistical significance was defined as \( p<0.05 \).

4.3 **Results**

4.3.1 **Experiment 1: Low and high fat, calcium and vitamin D on metabolic measures and ACF promotion.**

In the first experiment we considered the effects of fat, calcium and vitamin D on measures of metabolic changes and ACF promotion. An initial three-way ANOVA showed that the two dietary vitamin D levels (0.025 and 0.25 IU/kcal) had no effect on the metabolic and promotional measures affected by dietary calcium and fat (Appendix B). The data for the two levels of vitamin D were therefore collapsed to evaluate the effect of dietary fat and calcium.
Table 4.1. The effect of dietary calcium and fat on measures of metabolic changes and colon carcinogenesis.

<table>
<thead>
<tr>
<th>Factor Measured</th>
<th>Low Fat Low Calcium</th>
<th>Low Fat High Calcium</th>
<th>High Fat Low Calcium</th>
<th>High Fat High Calcium</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic Measures</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final Weight (g)</td>
<td>343 ± 3</td>
<td>336 ± 3</td>
<td>364 ± 4</td>
<td>372 ± 6</td>
<td>**</td>
</tr>
<tr>
<td>Food intake (kcal/day/rat)</td>
<td>67.1 ± 2.01</td>
<td>62.8 ± 1.07</td>
<td>64.7 ± 1.10</td>
<td>71.9 ± 1.12</td>
<td>*</td>
</tr>
<tr>
<td>Area Under the Curve (mM glucose x h)</td>
<td>14.5 ± 0.27</td>
<td>14.5 ± 0.31</td>
<td>15.5 ± 0.26</td>
<td>15.9 ± 0.36</td>
<td>**</td>
</tr>
<tr>
<td>Insulin (pmol/L)</td>
<td>450 ± 51</td>
<td>526 ± 64</td>
<td>469 ± 85</td>
<td>570 ± 69</td>
<td>*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.33 ± 0.07</td>
<td>1.17 ± 0.09</td>
<td>1.40 ± 0.10</td>
<td>1.24 ± 0.09</td>
<td>**</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>173 ± 6.41</td>
<td>183 ± 7.58</td>
<td>191 ± 7.52</td>
<td>180 ± 7.62</td>
<td></td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>0.41 ± 0.02</td>
<td>0.37 ± 0.02</td>
<td>0.69 ± 0.03</td>
<td>0.63 ± 0.03</td>
<td>*</td>
</tr>
<tr>
<td>Promotion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACF Size (ACI/ACF)</td>
<td>2.36 ± 0.05</td>
<td>2.49 ± 0.05</td>
<td>2.45 ± 0.05</td>
<td>2.89 ± 0.06</td>
<td>** ** *</td>
</tr>
<tr>
<td>ACF Number (ACF)</td>
<td>124 ± 6.6</td>
<td>153.3 ± 7.2</td>
<td>126.3 ± 8.1</td>
<td>166.4 ± 7.7</td>
<td>*</td>
</tr>
</tbody>
</table>
The results for the metabolic measures are shown at the top of Table 4.1. Food consumption was highest with the animals on the high fat, high calcium diet. High fat diets increased caloric intake, as did high calcium only within the high fat groups. Final weight was higher with the high fat diet but was unaffected by dietary calcium. Area under the oral glucose tolerance curve (a.u.c.) were not significantly different in animals receiving 0.5 as compared with 5.0 g/kg diet of calcium. Consistent with previous studies, the high fat diet increased a.u.c. at both dietary calcium concentrations (Koohestani et al., 1997). Glucose and triglycerides were not affected by the calcium and fat in the diets. Insulin levels were reduced in animals on a low fat diet with 0.5 g/kg calcium as compared to animals on the high fat diet. Non-esterified fatty acids were significantly elevated in response to a high fat diet at both levels of dietary calcium.

The results for the ACF measures are shown at the bottom of Table 4.1. A 10-fold reduction in dietary calcium from 5.0 to 0.5 g/kg diet significantly reduced the number and size of ACF in animals on both the high fat and AIN76-low fat diets. The effect of fat on ACF size was seen at 5.0 g/kg of calcium where animals on the high fat had significantly larger ACF than the low fat controls.

4.3.2 Experiment 2: Graded doses of calcium on metabolic and promotional measures in animals on low and high fat diets.

The results of experiment 1 indicated that ACF promotion was increased
when the calcium concentration was increased from 0.5 to 5 g/kg diet. To
determine whether, and at what concentration, increased calcium decreased
ACF promotion, the range of dietary calcium levels in the second experiment was
extended to 0.5, 1.5, 5 and 15 g/kg.

The results for the metabolic measures are shown in the upper panels of
Figure 4.2. There was a significant increase in food intake in animals on 15 g/kg
of calcium compared to animals receiving 0.5, 1.5 and 5 g/kg calcium with the
low fat diet. This difference was not seen in animals on the high fat diet.
Difference in food intake between the high fat and low fat groups was statistically
significance across all calcium concentrations but did not reach statistical
significance between groups at each individual calcium level as they had in
Experiment 1. The increase in dietary fat consistently resulted in a significant
increase in final weight across all dietary calcium levels. There were no
significant changes in final weight between 0.5 to 5 g/kg of calcium, however
there was a significant reduction in the final weight of animals consuming 15 g/kg
dietary calcium. With calcium increased above 1.5 g/kg there was a consistent
increase in fecal weight with the calcium content of the diet. The effect was most
marked with the high fat diets but a significant elevation in fecal weight between
the low and high fat groups was seen at only 5 g/kg of calcium. Difference in
a.u.c. between the high fat and low fat groups did not reach statistical
significance as they had in Experiment 1 (p = 0.056). Calcium and fat also had no
significant effect on glucose and triglyceride levels and the differences in insulin
levels did not reach statistical significance.
Figure 4.2. The effect of dietary calcium as calcium dibasic phosphate on metabolic and promotional measures of colon carcinogenesis in F344 rats. Different letters represent statistical significance, p<0.05. Difference between the high and low fat groups were assessed by a two-way ANOVA followed by t-tests, where * represents statistical significance, p<0.05.
Figure 4.2 continued. The effect of calcium and fat on metabolic and promotion measures.
The results for ACF size and number are shown in the bottom panel of Figure 4.2. Consistent with the results of experiment 1, there was a general increase in ACF size with increasing level of calcium in both the low and high fat groups. The same pattern was seen with ACF number with the low fat diet. By contrast there was a marked reduction in ACF number in the high fat diet when calcium increased from 5 to 15 g/kg. This result is illustrated in greater detail in Figure 4.3, which shows the distribution of ACF sizes (1-2, 3-4 and 5+) low and high fat for the graded levels of calcium. Here it is again evident that the number of large (5+) crypts increase continuously with increasing calcium with the low fat diet, but increase to 5 then decrease at the 15 g/kg calcium level.
Figure 4.3. The effect of dietary calcium and fat on ACF size. Groups were compared within 1-2, 3-4 and 5+ AC/ACF where different letters represent statistical significance, p≤0.05.
4.4 Discussion

Consistent with the widely observed reduction of colon promotion with increased calcium, we observed a reduction of large ACF and ACF number with animals on a high fat diet when dietary calcium, given as calcium dibasic phosphate, was increased from 5 to 15 g/kg (Pereira et al., 1997, Pence and Buddingh, 1988, Wargovich et al. 1990, Sitrin et al., 1991). Also consistent with the prior observations of Li et al. (1998) we observed a peak in the curve for animals on the high fat diet. That is, increased calcium below 5 g/kg increased our measures of promotion, while calcium increased above this level decreased promotion. The reduction in promotion above 5 g/kg was not observed with animals on the low fat diet.

Also consistent with earlier studies, increasing dietary calcium resulted in increased fecal weight, especially in the animals on the high fat diet (Behling et al., 1990, Lupton et al., 1994). This reduced available energy in animals on the low fat diet led to a marked increase in caloric intake when calcium levels were increased from 5 to 15 g/kg. There was curiously no such increase with the animals on the high fat diet. Weight gain decreased progressively from 1.5 to 5, to 15 g/kg calcium, the difference being more marked for the animals on the high fat diet, presumably because these animals did not increase food intake to compensate for the increased loss of nutrients in the fecal stream. There were thus significant changes in metabolic measures as assessed globally by
measures of food intake, fecal loss and weight gain. However these marked changes were not reflected in significant consistent changes in the plasma levels of glucose, lipids or of the glucose tolerance curves. Possibly these spot measures were insufficiently sensitive to detect the significant metabolic changes that resulted in changes of energy disposition. Calcium supplementation has been reported to reduce insulin resistance when measured in patients with a euglycemic, hyperinsulinemic glucose clamp (Sanchez et al., 1997). More sensitive approaches could well have identified changes in insulin resistance with dietary calcium change.

These studies were performed under conditions in which the calcium - phosphorous ratio was held at unity. Previous studies have differed in their levels of calcium supplementation and also likely of the dietary status of the animals or patients (Pereira et al., 1997, Pence and Buddingh, 1988, Wargovich et al. 1990, Sitrin et al., 1991). The character of the response to dietary calcium in this study suggests that results could differ considerably depending exactly on these factors. In contrast, dietary vitamin D under the conditions of this study had little if any effect on promotion or metabolic events.

Together these results thus support a model that demonstrates complex metabolic changes occur in response to increases in dietary calcium. As calcium concentrations are increased in a high fat diet, food is digested less efficiently allowing more energy to be left in feces and less available for absorption. As
shown here, this results in a reduction in weight gain even if there is an increase in caloric intake. In this study these metabolic effects of calcium were reflected on the promotion of ACF but were not accompanied by changes in circulating energy substrate levels or of the glucose tolerance curve.
CHAPTER 5: METABOLIC EFFECTS OF A HIGH FAT DIET IN THE F344 RAT

5.1 Introduction

McKeown-Eyssen and Giovannucci have pointed out that the promoting effect of the Western, high fat, high energy diet on colon carcinogenesis could be explained by metabolic processes. They both noted that these diets lead to insulin resistance and increased circulating insulin. They suggested that the elevated insulin could act as a growth factor and promoter, presumably by selectively increasing the growth of mutant cells with defective growth control mechanisms. (McKeown-Eyssen, 1994 and Giovannucci 1995). McKeown-Eyssen also noted that the diets that lead to insulin resistance also increase the levels of circulating lipids and she suggested that the high circulating energy level itself could act as a growth factor and promoter.

These hypotheses are not entirely original. It has been known for many years that insulin, glucose and triglycerides can all affect cell proliferation (Craven et al., 1988, Koenuma M et al., 1989, Cosio, 1995, Susini et al., 1998). There has also been evidence that colon cancer risk is associated with abnormalities in oral glucose tolerance tests and increased insulin resistance (Copeland et al., 1987, Kono et al., 1998, Kim 1998). What is new is the concept that metabolic processes are involved in the promotion of colon cancer and the results of a direct test of the hypothesis in a recent case control study that shows that colonic polyps and colon cancers are associated with increased levels of
insulin and triglycerides. (McKeown-Eyssen and the Toronto Polyp Prevention Group, 1999).

Experimental animal studies support a close association between high energy diets and both insulin resistance and promotion (Reddy et al., 1977, Kraegen et al., 1989). Koohestani et al., have recently examined the effect of diets differing in energy, n-3 fatty acids and fat on energy consumption, oral glucose tolerance, serum insulin, triglyceride and non-esterified fatty acids (NEFAs) and colon cancer promotion, assessed by the growth of aberrant crypt foci in F344 rats. They found that across the diets, dietary energy was closely associated with hyperinsulinemia, hypertriglyceridemia, a reduced glucose tolerance, and ACF promotion. They concluded, in parallel with the two hypotheses above, that promotion of carcinogenesis was a consequence of the elevated levels of circulating energy and/or elevated levels of insulin (Koohestani et al., 1998).

A limitation of all the previous assessments of circulating risk factors as possible promoters is that only single, “spot” measurements were made. As insulin and circulating energy levels vary greatly throughout the day it is difficult to assess their relative importance in the promotional process from single measurements. A further limitation is that no intracellular measures of cellular energy have been made. Triglycerides are known to increase in muscle and liver cells during the process of insulin resistance. An increase in other cell
populations could support the more general effects of the diet and metabolic processes throughout the body. These experiments have therefore compared the effect of two diets, high and low in dietary fat, known to differ in their effects on colon cancer promotion, for their effects on circulating insulin and energy substrates through the 24-h day. The effect of these diets on intracellular triglyceride in muscle, liver and spleen cells and in isolated colonic epithelial cells was also assessed.

5.2 Methods and Materials

5.2.1 Animals

Male Fischer 344 rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing approximately 180 g were housed individually in wire-bottom cages. The temperature and humidity were controlled at approximately 22°C and 50%, respectively. The room was maintained on 12-h dark/light cycles, with the dark cycle extending from 7 pm until 7 am. Tap water from an automatic system was provided ad libitum. Care of the animals conformed to the guidelines of the Canadian Council on Animals Care, and the protocol was approved by the University of Toronto Animal Care Committee.
5.2.2 Experimental Design

In both protocols (Fig. 5.1) the animals were acclimatized for 7 days on a Rodent chow diet (Ralston Purina International, Strathroy, Canada) and were then randomized by weight to high or low fat diet groups. In experiment 1, 60 animals, 30 in each group, remained on the diet for 3 weeks when they were divided into 5 groups of 6 and sacrificed after blood sampling under light anesthesia (Halothane) by cardiac puncture at 5 pm, 9 pm, 1 am, 5 am and 9 am. Plasma was obtained after the addition of EDTA (Miles Canada, Etobicoke, Canada) and centrifugation for 20 min at 1800 rpm, 4°C. The spleen, liver and soleus muscle were removed, coded and frozen immediately in liquid nitrogen from animals sacrificed at 9 am and 9 pm. The colon was also removed from these animals and colonocytes were collected as described below. Ten animals from each group also had an oral glucose tolerance test (OGTT) performed after 18 days on the diet.

In experiment 2, 22 animals, 11 in each group, were maintained on the low and high fat diets for three weeks when they were sacrificed for muscle, liver, spleen and colonic epithelial and blood samples.
Figure 5.1. A. Protocol scheme for experiment 1. Animals were acclimatized and then randomized into high fat and low fat groups. After 21 days on the diets, animals were randomized into 5 groups of 6 and sacrificed at the times indicated. Colonocytes were collected and intracellular TG measured using a glycerol-specific colorimetric kit. B. Protocol scheme for experiment 2. Animals were acclimatized and randomized onto the same diets as experiment 1. Upon sacrifice, colonic epithelial cells were removed and analyzed for intracellular lipids (TG and FA) using thin-layer and gas chromatography.
5.2.3 Diets

The low fat diet was based on the AIN76C (Bieri et al., 1977) diet where 18% energy was provided as protein (casein), 70% as carbohydrates, primarily cornstarch and 12% energy as fat in the form of corn oil. The high fat diet was balanced on an energy density basis with the low fat diet with respect to vitamins, minerals and protein. The high fat diet provided 60% energy as fat as beef tallow and corn oil. Both diets were provided by Dyets (Bethlehem, PA). Diet compositions are in appendix B.

5.2.4 OGTT and blood measures

For the OGTT, the animals were fasted for 7 h (7:00 am to 2:00 pm) and were then given an oral glucose gavage of 4 mg anhydrous glucose/g body weight. Blood glucose was measured from the tail vein using a glucometer (Medisense Canada, Toronto, Canada) prior to and 30, 60, 90 and 120 min after gavage. The total area under the glucose tolerance curve (a.u.c.) was calculated from 0 to 120 minutes and quantified using the trapezoid rule.(Wolever et al., 1991).

Glucose at sacrifice was measured by a glucose analyzer. Triglycerides and free fatty acids measured by colorimetric kits (kit 337 Sigma St. Louis, MA,
Kit 994-75409 Wako Richmond, VA) Insulin was measured using a radioimmunoassay (Rat insulin RIA kit; Linco Research, St. Charles, MO)

5.2.5 Collection of colonic epithelial cells

Colons were removed, washed with calcium and magnesium-free Hanks solution, cut longitudinally and then cut into 6-9 pieces. They were stored in Hanks + 0.03M EDTA for 1 h. Colonic epithelial cells were removed from the underlying mucosa by forcing the pieces of colon through a syringe. The cell suspension was centrifuged at 1800 rpm for 30 min, the liquid phase was discarded and the colonic epithelial cells were in the lower phase. Microscopic analysis revealed no evidence of contamination of the pellet of colonocytes by adipocytes or fibroblasts.

5.2.6 Measurement of intracellular triglycerides and free fatty acids

The tissue samples and colonic cell suspensions were homogenized using a dounce-type homogenizer. They were then subjected to a chloroform/methanol lipid extraction (Folch et al. 1957). The chloroform phase was evaporated under nitrogen and the triglyceride content of the remaining lipid layer was quantified using a triglyceride-specific colorimetric kit (kit 337, Sigma)
The intracellular triglycerides of colonic epithelial cells were also assessed by gas chromatography. Initial separation of the chloroform extract was accomplished with thin layer chromatography using petroleum ether:diethylether:acetic acid (80:20:1) solvent system, Whatman K6F silica gel 60 Å plates, 250 µm thickness and 0.02% 2'-7'dichloroflurescein in methanol spray. The triglyceride and free fatty acids phases were methylated as described by Morrison et al (1964) and subjected to gas chromatography, Hewlett Packard HP5890 (Palo Alto, CA) with 7673A auto-sampler and flame ionization detector. The column used was DB-23 30 x 0.32mm ID x 25 µm film by J & W Scientific (Folsom, CA). The temperature program was as follows: 50°C for 2 minutes, ramp A - 10°C per minute to 180°C; hold for 5 minutes, ramp B - 4°C per minute to 230°C; hold 2.5 minutes. The injection port temperature was 220°C and the detector temperature was 240°C. Integrated areas were compared with free fatty acid standard (Sigma-Aldrich) and triglyceride standard (Nuchek Prep, Elysian, MN).

5.2.7 Statistical Measures

The descriptive data was presented as mean ± SEM, except ratios of between high fat and low fat groups over the dark cycle which were reported as mean ± SD. Differences between the groups was verified using a one-way ANOVA. A modified two-tailed Students t-test using pooled error variances was
used to determine differences between means of diurnal measures and intracellular triglycerides.

5.3 Results

5.3.1 Experiment 1: Effect of a High Fat Diet on Plasma Energy Substrates over 24-hours and Intracellular Triglycerides

After three weeks on a high fat diet there was a significant increase in weight gain and insulin resistance as assessed indirectly using an oral glucose tolerance test and quantified as area under the curve (a.u.c.). High fat feeding resulted in a significant elevation in weight gain, 33.1 g versus 29.1 g in the low fat group (p=0.02). As shown in figure 5.2, animals on the high fat diet demonstrated an elevation in a.u.c., 15.4 ± 0.3 mM/hour as compared with low fat controls of 14.3 ± 0.3 mM/hour (p=0.02).

Results of measured plasma substrates are shown in figure 5.3. Energy intake was increased in animals on the high fat diet during the dark cycle, but lower during the light cycle. The most marked effects of the high fat diet on average values over 24 h are seen as a 1.82 ± 0.23 (p<0.001) fold increase in circulating NEFA. Triglyceride increased 1.29 ± 0.13 fold. An apparent 1.28 ± 0.15 fold increase in insulin levels did not reach statistical significance (p=0.035). Glucose concentrations remained unchanged. Together, the effects of the high
Figure 5.2. Experiment 1. Oral glucose tolerance test for animals on a high fat diet versus a low fat diet. Values reported as means ± SEM. Tail vein blood glucose was measured before and 30, 60, and 120 minutes after a glucose gavage (4 mg/g body weight).
Figure 5.3. Experiment 1. The effect of a high fat diet on metabolic measures assessed over 24 hours. Dark bar denotes the dark cycle when animals are most likely to be feeding. Statistical significance denoted as *, p≤0.05.
fat diet on all these measures were more pronounced through the dark cycle when the animals are feeding. Here, high fat feeding resulted in a $2.16 \pm 0.20$ (p<0.001), $1.31 \pm 0.04$ (p=0.10) and $1.50 \pm 0.08$ (p=0.05) fold increase in circulating NEFA, triglycerides and insulin, respectively (reported as mean ± SD).

The elevations in circulating lipids are reflected inside cells as increases in intracellular triglycerides of liver, spleen, muscle and colon samples as assessed by a colorimetric-specific kit, depicted in figure 5.4. These elevations due to the high fat diet were consistent in all tissue samples, however only reached statistical significance in liver and spleen tissue taken at 9 am. Intracellular triglyceride concentrations ranged from 0.8 mg triglyceride in the colon cells to 14 mg of triglyceride in liver per gram of tissue or cell suspension.

5.3.2 Experiment 2: Metabolic Measures and Intracellular Lipids

Gas chromatography was used to assess intracellular lipids in order to confirm results found using the colorimetric kit. Results of a high fat diet at 3 weeks on metabolic measures are shown in table 5.1. As seen in the left panel, the high fat diet lead to elevations in weight gain, food intake and plasma triglycerides at 3 weeks. Intracellular triglyceride and free fatty acids in colonocytes are shown in figure 5.5 and the right panel of table 5.1. Consistent with experiment 1, the high fat resulted in elevated intracellular triglyceride as well as increases in intracellular free fatty acids. Values ranged from 0.2 to 4.5
Table 5.1 Experiments 1 & 2. The effect of a high fat diet on metabolic measures and intracellular lipids. Metabolic measures for both experiments were assessed at 9 pm. Experiment 1 used a glycerol-specific colorimetric assay and experiment 2 used gas chromatography for determination of intracellular triglycerides and fatty acids (experiment 2 only). Different letter denotes statistical significance of p≤0.05.

<table>
<thead>
<tr>
<th>DIETARY GROUP</th>
<th>METABOLIC MEASURES</th>
<th>INTRACELLULAR ENERGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight Gain (g)</td>
<td>Glucose (mg/dL)</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exp't 1</td>
<td>Low Fat</td>
<td>29.2 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>High Fat</td>
<td>33.1 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Exp't 2</td>
<td>Low Fat</td>
<td>34.3 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>High Fat</td>
<td>38.5 ± 2.8</td>
</tr>
</tbody>
</table>
Figure 5.4. Experiment 1. The effect of a high fat diet on intracellular triglycerides of the liver, spleen, muscle and colonic epithelial cells using a glycerol-specific enzymatic method. Statistical significance denoted as *, p≤0.05.
Figure 5.5. Experiment 2. The effect of a high fat diet on intracellular triglycerides and fatty acids in colonic epithelial cells quantified using gas chromatography. Statistical significance denoted as *, p≤0.05.
mg lipid/g cell suspension. Gas chromatography yielded values approximately 2 fold higher than values obtained using the colorimetric kit.

5.4 Discussion

The purpose of this study was to examine two possible ways in which a high fat diet might affect colon carcinogenesis process, by increasing circulating insulin or by increasing circulating energy substrates. In the comparison of the effects of high versus a low fat diet over a 24-hour period, the factor most markedly affected was circulating non-esterified fatty acids (NEFA) which were increased almost two-fold. Triglycerides and insulin were elevated to a lesser extent, glucose was not affected at all. The elevations were most pronounced over the dark cycle when the animals are feeding and were not readily discerned during the period the animals were not eating. The increase in the circulating NEFA was reflected by elevations in intracellular triglycerides in both the dark and light cycle. This effect appeared to be similar in tissues from several organs, the liver, muscle, spleen and colon cells, although they only reached statistical significance at both times in the spleen and during the light cycle in the liver. The results thus demonstrate that for this model of carcinogenesis, the effects of a high energy diet on intravascular NEFA and intracellular triglycerides are consistent with an important role of intracellular energy pathways in the carcinogenesis process. However, they do not rule out a possible effect of elevated insulin on the process.
The 24 h intravascular metabolic measures are consistent with an earlier study in the rat (De Gasquet et al., 1977, Iwashita et al., 1996). These investigators also observed that a high fat diet increased circulating NEFA and triglycerides to a greater degree than insulin. The effects of other diets that affect caloric intake or of exercise on 24-h metabolic measures have not been reported. Studies directed to identifying the early steps in the development of insulin resistance have also found that high fat diets or direct intravenous injections of triglyceride lead to insulin resistance and increased intracellular triglycerides in liver and muscle cells (Kraegen et al., 1991, Boden et al., 1991).

The mechanism by which triglycerides inside the cell are thought to influence insulin resistance may be through the balance between free fatty acids and glucose utilization described by Randle and colleagues (Randle et al., 1963). Alternatively, a more broadly based mechanism suggests that elevations in glucose and lipids may initiate a cascade of second messengers such as diacylglycerol and calcium. Elevations in glucose provide substrates for the production of malonyl-coA, which acts to inhibit fatty acid oxidation by preventing fatty acid entry into the mitochondria. This results in an accumulation of long-chain coAs - intermediates in fatty acid metabolism. It is this elevation in long-chain coAs which is postulated to induce insulin resistance in some cell types (pancreatic B-cells), and perhaps affect carcinogenesis through alterations in proliferation and differentiation in other cell types (Prentki and Corkey, 1996).
Together the results of this study support the hypothesis that it is increases in energy availability that may be the underlying factor relating insulin resistance and carcinogenesis. The high risk diet increases circulating NEFA and TG, particularly postprandially, and the increased circulating energy substrates lead to increases in intracellular energy as triglycerides. In the liver and muscle this may lead to effects on glucose transport and the development of insulin resistance. In the colon, and perhaps other epithelial organs, the excess energy availability may affect cell cycle control in some way and lead to the promotion of initiated cells.

Although diurnal variations of these circulating energy substrates are greatly influenced by diet, most experimental and clinical assessments of these measures are taken after a period of fasting. (De Gasquet et al., 1977, Frayn et al., 1998). The results of this study suggest that the metabolic response to high risk diets will be most striking in the postprandial period. Of course, measurements during this period can show more variability and pose practical difficulties.
Chapter 6
CHAPTER 6: GENERAL DISCUSSION

6.1 Introduction

As outlined in chapter 3, McKeown-Eyssen and Giovannucci hypothesized that the development of insulin resistance and colon cancer promotion may be related. Together, they suggested that there are at least two underlying factors that may be responsible, hyperinsulinemia which may act to promote tumor growth, and/or increased availability of energy substrates such as triglycerides and NEFA which may provide energy to encourage proliferation (McKeown-Eyssen 1994, Giovannucci 1995). Observations by Koohestani et al. identified a strong correlation between energy intake and weight gain with ACF promotion – a correlation stronger than that of fasting and postprandial insulin and ACF promotion, and the same as that of postprandial TG and ACF promotion – implying that energy substrate availability may be important. Following from these observations, this thesis hypothesized that elevations in energy availability may be important in colon carcinogenesis. The objectives of this work were to (1) determine whether a dietary factor which alter promotion and insulin resistance, namely calcium, affect energy availability in the same manner, and (2) to characterize the diurnal variations of energy substrates and see to what extent these variations manifest themselves inside colonic epithelial cells.

The results of the experiments conducted to address these objectives are found in chapters 4 and 5. Colon cancer development was assessed by the size
and number of aberrant crypt foci (ACF) and the oral glucose tolerance test was used as an indirect measure of insulin resistance. To answer the first objective, the effect of diets differing in dietary calcium, vitamin D and fat on ACF promotion and area under the OGTT (a.u.c.) were compared. As outlined in chapter 3, supplemental calcium has been related to a reduced risk of diabetes mellitus and colon cancer, making dietary calcium an attractive agent to consider the role of energy in light of the McKeown-Eyssen/Giovannucci hypothesis. Dietary calcium was found to alter both promotion and energy availability as assessed by energy intake, weight gain and fecal mass. The second objective was addressed by comparing the effects of diets low and high in fat on circulating insulin, glucose, triglycerides and NEFA. Insulin, triglycerides and NEFA concentrations were elevated as a result of high fat feeding, moreover, these elevations were more pronounced over the dark cycle when animals are most likely to be feeding. The high fat diet affected NEFA concentrations to the greatest extent and the increase in intravascular energy availability was manifested as elevations in intracellular triglycerides of various tissues and of colonic epithelial cells.

6.2 Overview of the Results

The results of this research support the notion that energy availability may be important in tumor development and may explain the relationship between insulin resistance and ACF promotion. In the first experiment it was shown that dietary calcium influences promotion. Three observations raise questions as to
the manner in which dietary calcium affects tumor promotion. The first was observations of a multiphasic effect of calcium on ACF number in the presence of a high fat diet, where low and high calcium concentrations as compared to the levels found in the standard AIN76C diet reduce ACF number. The second result to note was the apparent lack of effect of a wide range of calcium concentrations on a.u.c, albeit an indirect measure of insulin resistance. The third observation was the dramatic decrease in energy availability as dietary calcium concentrations increased, demonstrated by striking decreases in weight gain and increases in fecal loss. Together these results suggest that calcium acts to decrease the amount of food absorbed perhaps by complexing with dietary fat and contributing to its excretion, not allowing for absorption. These observations are consistent with the hypothesis of Newmark et al., 1994 who suggested that calcium forms complexes with bile acids and fatty acids which can irritate the colon, thereby reducing compensatory hyperproliferation. This is also consistent with the notion that energy from the diet is important in cancer risk, where several studies have shown that calorie restricted diets reduce colorectal cancer promotion in addition to body weight (Kumar et al., 1990). Although dietary calcium affected promotion and indicators of energy availability, there were no significant changes in circulating triglycerides, NEFA or a.u.c.

In order to better understand the potential role of circulating energy substrates in colon tumor promotion, the second group of experiments sought to delineate diurnal variations of insulin, glucose, triglycerides and free fatty acids
and the effect of a high saturated fat diet on these measures. Results showed that the high fat diet elevated NEFA levels 2-fold, where triglycerides and insulin were affected to a lesser extent. In addition, these elevations were more pronounced in the dark cycle when animals are feeding. The increase in intravascular energy substrates were reflected inside tissues as elevations in intracellular triglycerides. Increases in intracellular triglycerides of the liver, spleen, muscle (consistent with insulin resistance), and for the first time colon cells, was shown. When tested using a more sensitive assay gas chromatography, elevations in intracellular lipid of colonocytes were confirmed. The results obtained when using gas chromatography were approximately 2-fold higher than those found using the glycerol-specific colorimetric kit. Although several studies have shown a correlation between these two methods in the measurement of free fatty acids, there are other investigators who have found as much as 27% bias (Brunk et al., 1981, Mulder et al., 1983). In addition, it is difficult to compare these two methods directly as the samples were obtained from two different groups of animals.

The apparent discrepancy as to the potential importance of energy substrates, namely blood triglycerides in tumor promotion between the two sets of experiments may be explained in two ways. The first was the assessment of circulating energy substrates, where measurements in the calcium studies were taken only at one time point. As shown in the second study, there are wide diurnal variations and so a 24-hour profile is ideal in determining the role of these
circulating measures. Secondly, the latter study demonstrated that circulating NEFA rather than triglycerides appear to be more indicative of changes of intravascular energy availability.

6.3 Conclusions

McKeown-Eyssen and Giovannucci suggested that diet might be affecting promotion and insulin resistance in the same manner. Together they postulated that the relationship between these etiologies might be through hyperinsulinemia and/or increases in energy availability. This thesis supports the notion that energy availability may be important. There were striking effects of dietary calcium on weight gain and fecal loss, although the changes in circulating triglycerides measured at one time point, as well as a.u.c. were not as apparent. The second study confirmed that there are significant diurnal variations in NEFA, triglycerides and insulin over 24-hour period, and that the effects of a high risk, high fat diet are most impressive in postprandial period. The elevations in intravascular NEFA were reflected inside several tissues and colonic epithelial cells. Together these results suggest that energy availability may be important in (1) explaining the relationship between insulin resistance and colon cancer promotion, (2) that energy availability may play an important role in contributing to cellular defects which can ultimately lead to tumor development and (3) supports the idea that diet ilicits its effect systemically, not just locally. Further experiment studies are needed to investigate the mechanism(s) by which
elevations of intravascular energy substrates altered by dietary factors can contribute to cancer growth.

6.4 Future Work

Two kinds of studies can be considered for the future. The first are those investigating the nature of cellular energy and promotion and the second are those surrounding the role of calcium and promotion.

6.4.1 Intracellular Triglycerides

This work demonstrated an elevation in intracellular lipids as a result of a high fat diet. Studies considering the way in which these lipids may contribute to cellular growth and carcinogenesis would provide insight as to how dietary fat affects colon cells, these may include studies on how controlled elevations of circulating NEFA affect proliferation of the colonic epithelial cells. In addition, the effect of other dietary factors such as calcium on intracellular lipids can be investigated.

6.4.2 Calcium and Promotion

The role of calcium and colorectal cancer remains somewhat controversial. As outlined in chapter 3, there is a body of literature that has
reported a protective effect of supplemental calcium in both epidemiological and experimental models. This study demonstrated an inverse effect of calcium on promotion and a multiphasic response in the presence of a high fat diet. This infers that two types of mechanistic studies would be useful in order to understand these observations. The first are mechanistic studies designed to understand the interaction between dietary calcium and fat, and the second are studies which would investigate the manner in which dietary calcium alters cellular processes such as cell cycle control. Both sets of investigations would be key in being able to recommend preventative strategies.
Chapter 7
CHAPTER 7: REFERENCES


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Appendix A

Results of a three-way ANOVA for the effects of calcium, vitamin D and fat on markers of metabolic changes and ACF promotion
Appendix A:

Results of a three-way ANOVA for the effects of calcium, vitamin D and fat on markers of metabolic changes and ACF promotion (p-values)

<table>
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<th>Food intake</th>
<th>Weight gain</th>
<th>A.U.C.</th>
<th>insulin</th>
<th>glucose</th>
<th>TG</th>
<th>NEFA</th>
<th>ACF size</th>
<th>ACF number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (F)</td>
<td>0.073</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.010</td>
<td>0.250</td>
<td>0.250</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.427</td>
</tr>
<tr>
<td>Calcium (C)</td>
<td>0.234</td>
<td>0.869</td>
<td>0.606</td>
<td>0.376</td>
<td>0.184</td>
<td>0.184</td>
<td>0.092</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Vitamin D (D)</td>
<td>0.320</td>
<td>0.716</td>
<td>0.559</td>
<td>0.910</td>
<td>0.907</td>
<td>0.907</td>
<td>0.921</td>
<td>0.391</td>
<td>0.051</td>
</tr>
<tr>
<td>F x C</td>
<td>&lt;0.001</td>
<td>0.103</td>
<td>0.476</td>
<td>0.050</td>
<td>0.313</td>
<td>0.313</td>
<td>0.776</td>
<td>0.004</td>
<td>0.360</td>
</tr>
<tr>
<td>F x D</td>
<td>0.095</td>
<td>0.773</td>
<td>0.084</td>
<td>0.632</td>
<td>0.117</td>
<td>0.117</td>
<td>0.726</td>
<td>0.655</td>
<td>0.690</td>
</tr>
<tr>
<td>C x D</td>
<td>0.125</td>
<td>0.095</td>
<td>0.060</td>
<td>0.125</td>
<td>0.809</td>
<td>0.809</td>
<td>0.884</td>
<td>0.153</td>
<td>0.338</td>
</tr>
<tr>
<td>F x C x D</td>
<td>0.550</td>
<td>0.738</td>
<td>0.056</td>
<td>0.317</td>
<td>0.573</td>
<td>0.573</td>
<td>0.858</td>
<td>0.248</td>
<td>0.720</td>
</tr>
</tbody>
</table>

These results were calculated using SPSS version 3.0, significance defined as \( p<0.05 \). These p-values demonstrate that vitamin D had no significant effect on endpoints measured allowing for data to be collapsed and pooled into 4 groups varying in levels of fat and calcium.
Appendix B

Diet Compositions
Table B.1: Diet composition for experiment (g/100 g diet). The effect of calcium, vitamin D and fat on markers of insulin resistance and ACF promotion.

<table>
<thead>
<tr>
<th>Fat</th>
<th>1.1</th>
<th>1.2</th>
<th>1.3</th>
<th>1.4</th>
<th>1.5</th>
<th>1.6</th>
<th>1.7</th>
<th>1.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>HF</td>
<td>HF</td>
<td>HF</td>
<td>HF</td>
</tr>
<tr>
<td>Calcium (g/100 g diet)</td>
<td>5.0</td>
<td>5.0</td>
<td>0.5</td>
<td>0.5</td>
<td>5.0</td>
<td>5.0</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin D (IU/100g diet)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>10</td>
<td>100</td>
<td>10</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>Protein (casien)</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>24.6</td>
<td>24.6</td>
<td>24.6</td>
<td>24.6</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Fat (corn oil)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Fat (beef tallow)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Starch (corn starch)</td>
<td>61.5</td>
<td>51.6</td>
<td>62.7</td>
<td>62.8</td>
<td>18.7</td>
<td>18.8</td>
<td>20.8</td>
<td>20.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Fiber (cellulose)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.37</td>
<td>1.37</td>
<td>1.37</td>
<td>1.37</td>
</tr>
<tr>
<td>Vitamin D3 (1000IU)</td>
<td>0.09</td>
<td>0.09</td>
<td>0.123</td>
<td>0.123</td>
<td>0.123</td>
<td>0.123</td>
<td>0.123</td>
<td>0.123</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>1.575</td>
<td>1.575</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
<td>2.16</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>TBHQ</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td>Total Weight</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Protein (kcal/100g diet)</td>
<td>73.2</td>
<td>73.2</td>
<td>73.2</td>
<td>73.2</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
</tr>
<tr>
<td>Complex CHO (kcal/100g diet)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>16.4</td>
<td>16.4</td>
<td>16.4</td>
<td>16.4</td>
</tr>
<tr>
<td>Simple CHO (kcal/100g diet)</td>
<td>266.2</td>
<td>266.5</td>
<td>271.0</td>
<td>271.3</td>
<td>101.9</td>
<td>102.3</td>
<td>110.4</td>
<td>110.9</td>
</tr>
<tr>
<td>Fat (kcal/100g diet)</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>331.2</td>
<td>331.2</td>
<td>331.2</td>
<td>331.2</td>
</tr>
<tr>
<td>Energy (kcal/100g diet)</td>
<td>396.4</td>
<td>396.7</td>
<td>401.2</td>
<td>401.5</td>
<td>549.1</td>
<td>549.5</td>
<td>557.6</td>
<td>558.1</td>
</tr>
<tr>
<td>Calcium (mg/kcal)</td>
<td>1.3</td>
<td>1.3</td>
<td>0.12</td>
<td>0.12</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Phosphate (mg/kcal)</td>
<td>3.1</td>
<td>3.1</td>
<td>0.31</td>
<td>0.31</td>
<td>3.0</td>
<td>3.0</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>Vitamin D (IU/kcal)</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Note: Average fatty acid profile (mg FA/g diet) for selected high fat diets: Saturated 17.7, Monounsaturated 11.9, Polyunsaturated (PUFA) 5.5, n-3 PUFA 0.2 Low fat diets: Saturated 0.8, Monounsaturated 2.1, Polyunsaturated 5.0, n-3 PUFA 0.05

LF = low fat, HF = high fat
Table B.2: Diet composition for experiment 2 (g/100 g diet). The effect of a range of calcium levels and fat on markers of insulin resistance and ACF promotion.

<table>
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<tr>
<th>Fat</th>
<th>2.1</th>
<th>2.2</th>
<th>2.3</th>
<th>2.4</th>
<th>2.5</th>
<th>2.6</th>
<th>2.7</th>
<th>2.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (g/100 g diet)</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>LF</td>
<td>HF</td>
<td>HF</td>
<td>HF</td>
<td>HF</td>
</tr>
<tr>
<td>Protein (casien)</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>24.6</td>
<td>24.6</td>
<td>24.6</td>
<td>24.6</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Fat (corn oil)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Fat (beef tallow)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Starch (corn starch)</td>
<td>64.0</td>
<td>63.7</td>
<td>62.4</td>
<td>59.1</td>
<td>20.9</td>
<td>20.5</td>
<td>18.8</td>
<td>14.7</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>Fiber (cellulose)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1.37</td>
<td>1.37</td>
<td>1.37</td>
<td>1.37</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Calcium phosphate, dibasic</td>
<td>0.334</td>
<td>1.575</td>
<td>4.92</td>
<td>0.462</td>
<td>2.16</td>
<td>6.78</td>
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<td></td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
<td>0.27</td>
</tr>
<tr>
<td>TBHQ</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td>Total Weight</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Protein (kcal/100g diet)</td>
<td>73.2</td>
<td>73.2</td>
<td>73.2</td>
<td>73.2</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
</tr>
<tr>
<td>Complex CHO (kcal/100g diet)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>16.4</td>
<td>16.4</td>
<td>16.4</td>
<td>16.4</td>
</tr>
<tr>
<td>Simple CHO (kcal/100g diet)</td>
<td>276.0</td>
<td>274.6</td>
<td>269.7</td>
<td>256.3</td>
<td>111.0</td>
<td>109.2</td>
<td>102.4</td>
<td>86.0</td>
</tr>
<tr>
<td>Fat (kcal/100g diet)</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>331.2</td>
<td>331.2</td>
<td>331.2</td>
<td>331.2</td>
</tr>
<tr>
<td>Energy (kcal/100g diet)</td>
<td>406.16</td>
<td>404.84</td>
<td>399.88</td>
<td>386.52</td>
<td>558.2</td>
<td>556.36</td>
<td>549.56</td>
<td>533.16</td>
</tr>
<tr>
<td>Calcium (mg/kcal)</td>
<td>0.12</td>
<td>0.37</td>
<td>1.3</td>
<td>3.9</td>
<td>0.10</td>
<td>0.34</td>
<td>1.3</td>
<td>3.9</td>
</tr>
<tr>
<td>Phosphate (mg/kcal)</td>
<td>0.30</td>
<td>0.88</td>
<td>3.1</td>
<td>9.3</td>
<td>0.22</td>
<td>0.81</td>
<td>3.0</td>
<td>9.2</td>
</tr>
</tbody>
</table>

Note: Average fatty acid (mg FA/g diet) profile for selected high fat diets: saturated 13.1, monounsaturated 13.4, polyunsaturated (PUFA) 5.0 n-3 PUFA 0.2. For selected low fat diets: saturated 0.8, monounsaturated 2.1 polyunsaturated 5.0 (PUFA), n-3 PUFA 0.054 LF= low fat, HF = high fat
Table B.3. Diet composition for experiments 1 and 2 (g/100 g diet). The effect of a high fat diet on metabolic measures in the F344 rat.

<table>
<thead>
<tr>
<th></th>
<th>LF (g/100g)</th>
<th>HF (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>18</td>
<td>24.6</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Corn starch</td>
<td>64</td>
<td>20.86</td>
</tr>
<tr>
<td>Sucrose</td>
<td>5</td>
<td>6.8</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>6.8</td>
</tr>
<tr>
<td>Beef tallow</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3</td>
<td>4.1</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>3.5</td>
<td>4.8</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1</td>
<td>1.37</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
<td>0.27</td>
</tr>
<tr>
<td>TBHQ</td>
<td>0.001</td>
<td>0.007</td>
</tr>
<tr>
<td>TOTAL</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>% calories</td>
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<td>18</td>
</tr>
<tr>
<td>Protein</td>
<td>12</td>
<td>60</td>
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<td>Fat</td>
<td>64</td>
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<td>Complex CHO</td>
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<td>Simple CHO</td>
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<td>6</td>
</tr>
<tr>
<td>Total kcal/100g</td>
<td>401.5</td>
<td>552</td>
</tr>
</tbody>
</table>
Appendix C

The effect of a high fat diet on metabolic measures – 12 weeks
Appendix C

Experiment: The effect of a high fat diet on metabolic measures – 12 weeks

Background: Study 2 in chapter 5 considered the effect of 3 weeks of dietary intervention on circulating metabolic measures and intracellular triglycerides and fatty acids. This experiment is an extension of this study considering the effects of 12 weeks on a high fat diet on the same endpoints.

Objective: To determine the effect of 12 weeks of a high fat diet on metabolic measures and intracellular lipids of colonic epithelial cells.

Methods and Materials: 22 male F344 rats were acclimatized for 7 days and then randomized by weight into two dietary groups, one receiving a low fat diet (based on the AIN76C diet) and the other group receiving a high fat diet (described in appendix B). After 12 weeks on the diet, animals were sacrificed at 9 pm and blood and colonic epithelial cells were collected. Blood glucose, triglyceride and non-esterified fatty acids were measured as described. Intracellular lipids were separated using thin-layer chromatography and analyzed using gas chromatography.

Results: As seen in table C.1, there was a significant increase in weight gain in animals on the high fat diet. Plasma glucose levels appeared to be unaltered by the high fat diet. NEFA and triglyceride concentrations were elevated in animals on the high fat, however only reached statistical significance for NEFA levels. Intracellular fatty acids were elevated in animals on the high fat diet as compared to the low fat controls, however this did not reach
statistical significance. Interestingly, intracellular triglycerides of colonic epithelial cells were elevated in both the low and high fat diet suggesting complex homeostatic mechanisms in response to a prolonged period on a high fat diet, see figure C.1 and table C.1.

**Figure C.1:** The effect of a high fat diet on intracellular triglycerides (TG) and fatty acids (FA) of colonic epithelial cells as measured by gas chromatography.
Table C.1: The effect of a high fat diet on metabolic measures and intracellular energy in F344 rats. Animals remained on diets for 12 weeks, intracellular lipids of colonic epithelial cells were separated using thin-layer chromatography and quantified using gas chromatography. Different letters denote statistical significance of $p \leq 0.05$.

<table>
<thead>
<tr>
<th>DIETARY GROUP</th>
<th>METABOLIC MEASURES</th>
<th>INTRACELLULAR ENERGY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weight Gain (g)</td>
<td>Glucose (mg/dL)</td>
</tr>
<tr>
<td>Low Fat</td>
<td>81.1 ± 8.4$^a$</td>
<td>10.4 ± 0.4</td>
</tr>
<tr>
<td>High Fat</td>
<td>109.7 ± 5.6$^b$</td>
<td>9.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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