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SUSCEPTIBILITY OF ZUCKER RATS TO
MAMMARY GLAND AND COLON CARCINOGENESIS

by

Winnie M. Lee

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Nutritional Sciences
University of Toronto

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SUSCEPTIBILITY OF ZUCKER RATS TO MAMMARY GLAND AND COLON CARCINOGENESIS

Master of Science, 1998
Winnie M. Lee
Graduate Department of Nutritional Sciences
University of Toronto

ABSTRACT

The objectives were to determine whether the Zucker rat strain is susceptible to breast and colon carcinogenesis, and whether the metabolic abnormalities of obese (fa/fa) rats increase their susceptibility towards carcinogenesis compared to lean (FA/FA or FA/fa) rats. Breast and colon cancer was initiated with 37.5mg/kg MNU at 50 days of age followed by two weekly doses of 15mg/kg AOM. Lean and obese rats were susceptible to developing both cancers. However, mammary and colon tumor incidence and multiplicity were higher in obese rats than in lean rats. DNA methylation levels showed that obese rats received a higher biologically effective MNU dose than lean rats, although AOM doses were comparable. Therefore, the results suggest that the Zucker rat strain is susceptible to breast and colon cancer. Furthermore, obese rats are more susceptible to colon carcinogenesis than lean rats, although the relative susceptibility of Zucker rats towards breast cancer requires further investigation.
ACKNOWLEDGEMENTS

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<td>ω-3</td>
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</tr>
<tr>
<td>ω-6</td>
<td>omega-6</td>
</tr>
<tr>
<td>4-AAP</td>
<td>4-aminoantipyrine</td>
</tr>
<tr>
<td>7-meG</td>
<td>7-methylguanin e</td>
</tr>
<tr>
<td>AB</td>
<td>alveolar bud</td>
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<tr>
<td>AC</td>
<td>aberrant crypt</td>
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<tr>
<td>ACF</td>
<td>aberrant crypt foci</td>
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<tr>
<td>AM</td>
<td>azomethane</td>
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<tr>
<td>AOM</td>
<td>azoxymethane</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>cp</td>
<td>corpulent</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>cytochrome P_{450}2E1</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[α]anthracene</td>
</tr>
<tr>
<td>DMH</td>
<td>1,2-dimethylhydrazine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>ESPA</td>
<td>N-ethyl-N-(3-sulfopropyl)m-anisidine</td>
</tr>
<tr>
<td>F344</td>
<td>Fischer 344</td>
</tr>
<tr>
<td>FAP</td>
<td>familial adenomatous polyposis</td>
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<td>FFA</td>
<td>free fatty acid</td>
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<tr>
<td>GK</td>
<td>glycerol kinase</td>
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<td>glycerol phosphate oxidase</td>
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<td>GLUT</td>
<td>glucose transporter</td>
</tr>
<tr>
<td>GRB-2</td>
<td>growth factor receptor bound protein-2</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HNPCC</td>
<td>hereditary nonpolyposis colorectal cancer</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>i.g.</td>
<td>intragastric</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IR</td>
<td>insulin receptor</td>
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<td>IRS-1</td>
<td>insulin receptor substrate-1</td>
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<tr>
<td>i.v.</td>
<td>intravenous</td>
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<tr>
<td>IVGTT</td>
<td>intravenous glucose tolerance test</td>
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<td>LE</td>
<td>Long-Evans</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>MCA</td>
<td>methylcholanthrene</td>
</tr>
<tr>
<td>MFO</td>
<td>mixed function oxidase</td>
</tr>
<tr>
<td>MNU</td>
<td>N-methyl-N-nitrosourea</td>
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<td>MX</td>
<td>mineral mix</td>
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</table>
NaCl: sodium chloride
NIDDM: non-insulin dependent diabetes mellitus
ob: obese / leptin
OB-R: obese receptor or leptin receptor
OGTT: oral glucose tolerance test
PA: phytic acid
PBS: phosphate buffered saline
PEPCK: phosphoenolpyruvate carboxykinase
PUFA: polyunsaturated fatty acid
RIA: radioimmunoassay
s.c.: subcutaneous
SD: Sprague-Dawley
SDS: sodium dodecyl sulfate
SEM: standard error of the mean
STZ: streptozotocin
TBR: tumor bearing rat
TE: Tris-EDTA
TEB: terminal end bud
TG: triglyceride
Tris: tris[hydroxymethyl]amino-methane
VLDL: very low density lipoprotein
VMH: ventromedial hypothalamus
VX: vitamin mix
WF: Wistar Furth
CHAPTER ONE

Introduction and Literature Review
1. Introduction and Literature Review

1.1 Introduction

Mammary cancer and colon cancer are two of the most common causes of morbidity and mortality in Western countries (NCIC, 1998). Migrant studies have shown that both cancers are highly influenced by environmental factors (Haenszel, 1961; Kelsey et al., 1993; McMichael et al., 1988; Wynder et al., 1967). Dietary fat and excess caloric intake/obesity has been shown to play an important role in the etiology of both cancers (Aldercreutz, 1990; Potter et al., 1993). In addition, an epidemiological study by Seely and Horrobin (1983) has shown a positive relationship between breast cancer rates and sugar consumption. Similarly, several other epidemiological studies have shown refined sugars as a major dietary risk factor for colorectal cancer (Centonze et al., 1993; La Vecchia et al., 1993; Tuyns et al., 1987). Therefore, refined dietary carbohydrate may be an important dietary factor in breast and colon carcinogenesis. Seely and Horrobin (1983) hypothesized that excessive insulin secretion in response to sugar consumption results in a hormonal milieu that predisposes to mammary tumor development. Recently, Kazer (1995) and Stoll (1996) have also proposed that insulin resistance may be a risk factor for breast cancer since the factors that lead to insulin resistance are also those associated with breast cancer development.

McKeown-Eyssen (1994) and Giovannucci (1995) have suggested that the hyperinsulinemia and insulin resistance characteristic of non-insulin dependent diabetes (NIDDM) may be a mediator of the colon cancer promotion observed with dietary risk
Chapter One

factors, such as fat. Elevated insulin levels as a result of insulin resistance may act as a growth factor for cellular proliferation. Animal studies and in vitro work (Corpet et al., 1997; Heuson and Legros, 1970-1972; Heuson et al., 1967, 1972; Tran et al., 1996) have further supported the promoting effect of insulin and/or insulin resistance on both breast and colon cancer development. Therefore, hyperinsulinemia and insulin resistance appear to be important risk factors for both breast and colon cancer. However, the mechanism(s) by which insulin, insulin resistance and dietary risk factors, such as dietary fat and energy intake, are able to modulate the development of both breast and colon cancer are still unclear.

The aim of the work presented in this thesis was to examine the susceptibility of the Zucker rat strain towards breast and colon cancer. Furthermore, since the obese fafa Zucker rat is a well-characterized model of NIDDM and obesity that exhibits hyperinsulinemia, insulin resistance, and hyperphagia (Bray, 1977; Zucker, 1972), it was hypothesized that these metabolic abnormalities predisposes them to increased susceptibility to both mammary gland and colon carcinogenesis. A dual organ protocol (Shivapurkar et al., 1996) in which breast and colon cancer were initiated with the mammary-specific carcinogen, N-methyl-N-nitrosourea (MNU) and the colon-specific carcinogen, azoxymethane (AOM) was used to study the susceptibility of the Zucker rat strain towards carcinogenesis.
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1.2 Literature Review

1.2.1 Trends in Breast and Colon Cancer Incidence

Breast and colon cancer are both leading causes of morbidity and mortality in Western countries (NCIC, 1998). The highest rates of both cancers are found in the developed world. Colon cancer is the third most common cancer and cause of death in the United States and Canada while breast cancer ranks first among women (NCIC, 1998). Internationally, breast and colon cancer incidence and mortality rates have been shown to differ more than five-fold (IARC, 1987) and ten-fold, respectively (Boyle et al., 1985). Migrant studies have indicated that both breast and colon cancer rates increase in those migrating from low to high-incidence areas (Haenszel et al., 1968; Kelsey et al., 1983; McMichael et al., 1988). These epidemiological observations suggest that the development of both breast and colon cancer are influenced by environmental factors.

It is known that a genetic component exists for both cancers. Women with a first degree relative with breast cancer have a 2-3 fold higher risk of developing the cancer themselves (Kelsey, 1979). The recently identified tumor suppressor genes, BRCA 1 and BRCA 2, account for most of the inherited cases (~5%) of breast and ovarian cancer (King, 1992). Similarly, it has been established that some individuals have a genetic predisposition towards the development of colon cancer (Cannon-Albright et al., 1988; Vogelstein et al., 1988). There are several rare genetic syndromes, such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), which are accompanied by an excess risk of colon cancer (Kinzler and Vogelstein, 1996).
Recent evidence has suggested that predisposition to developing FAP involves a genetic defect in the APC gene which functions as the "gatekeeper" in the multi-step process of colon cancer development (Kinzler and Vogelstein, 1996). In contrast, the defect in HNPCC affects tumor progression via mutations in DNA mismatch repair genes, including hMSH2, hMLH1, and hPMS2 (Kinzler and Vogelstein, 1996).

While these genes may play a role in sporadic and non-inherited cases of colon cancer and breast cancer, epidemiological evidence has strongly suggested that lifestyle factors play an important role in the development of both cancers. In particular, both cancers have been shown to be strongly associated with dietary factors. Dietary fat, energy intake, and refined carbohydrate intake have all been shown to be important dietary risk factors in the development of breast and colon cancer. However, these same factors are also associated with the development of hyperinsulinemia and insulin resistance, which has shown to be positively associated with breast and colon cancer risk. Therefore, to understand the extent to which these dietary factors are associated with insulin and insulin resistance in mammary and colon carcinogenesis, a discussion of each risk factor will follow.

1.2.2 Dietary Risk Factors. Breast and Colon Cancer

1.2.2.1 Dietary Fat

International ecological studies have shown a strong correlation between dietary fat and breast cancer as well as colon cancer. In a comparison of incidence rates for 27 cancers in 23 countries and mortality rates for 14 cancers in 32 countries, Armstrong and
Doll (1975) showed that dietary fat, as determined by estimated per capita consumption rates, is highly correlated with both colon and breast cancer. The close relationship between dietary fat and breast or colon cancer seems to apply to animal fat. Rose (1986) showed a strong correlation between animal fat and age-adjusted breast cancer mortality rates for 30 countries in 1978-1979 (i.e. $r = 0.70$) although no correlation was observed between mortality rates and estimated vegetable fat intake (i.e. $r = 0.18$). Willet et al. (1990) have found in a prospective cohort study among 88,751 women, that the relative risk of colon cancer was positively associated with animal fat, after adjusting for total energy intake. Furthermore, the relative risk for the highest quintile as compared with the lowest quintile was 1.89 while no association was found for vegetable fat (Willet et al., 1990).

As a result of the strong evidence from descriptive epidemiological studies, excessive dietary fat has long been held as a risk factor for both breast and colon cancer. However, the analytical epidemiological evidence has been less consistent and has generally not supported the dietary fat-breast cancer hypothesis. Two population-based prospective cohort studies conducted by Hirayama (1978, 1979) examined the association between the intake of fat-containing foods and risk of breast cancer. Dietary information collected from 142,857 Japanese females showed that women who ate meat daily and who therefore consume relatively large amounts of fat, protein, and calories had a higher standardized mortality ratio for breast cancer than vegetarians. In contrast, Phillips and Snowdon (1983) were unable to find a relationship between the frequency of consumption of meat and the risk of death from breast cancer when they conducted a follow-up study.
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of Seventh-Day Adventists women in California. In the United States, the Nurses Health Study has been the largest cohort study examining the relationship between diet and breast cancer risk (Willet et al., 1987). Intake of dietary fat was measured using a semiquantitative food frequency questionnaire. After four years of follow-up, 601 of 89,853 women had developed breast cancer. However, the risk of developing the cancer was not associated with total caloric intake, total fat intake or saturated fat intake, in both the total study population and within menopausal strata. The absence of an association between dietary fat and breast cancer risk was also observed in a report of an eight year follow up in the Nurses' Health Study (Willet et al., 1992).

In a recent meta-analysis of studies of dietary fat and breast cancer risk, Boyd et al. (1993) reported that the relative risk for case-control studies that examined fat as a nutrient was 1.21, suggesting a positive, albeit weak, association. In this study, Boyd et al. (1993) did not partition fat intake into quintiles to analyze for relative risk. Rather, the partitions used were calculated based on those selected by the authors of the original studies. Howe et al. (1990), however, examined 12 case-control studies using a different method of partitioning fat intake and found a statistically significant, positive correlation between breast cancer risk and saturated fat intake in postmenopausal women (i.e. relative risk for highest versus lowest quintile, 1.46, P<0.0001). These differences in strength of association between breast cancer risk and dietary fat in a number of case-control studies further highlight the inconsistent nature of the epidemiological evidence to support dietary fat as a possible positive nutritional risk factor for breast cancer.
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In contrast to breast cancer, evidence from case-control studies have generally supported a positive association between dietary fat and colon cancer risk. Jain et al. (1980) conducted a case-control study in Canada with a total of 348 cases of colon cancer individually matched by sex, age, and neighbourhood of residence to 542 population controls and frequency matched to 535 hospital controls who had undergone an abdominal operation. In this study, saturated fat was highly correlated with colon cancer risk in both sexes, with evidence of a dose-response relationship (Jain et al., 1980). This striking positive association between colon cancer risk and dietary fat has been further shown in a number of other case-control studies (Miller et al., 1983; Bristol et al., 1985; Potter and McMichael, 1986; Graham et al., 1988; Kune and Kune, 1987). However, there have been a few exceptions to this strong correlation. Tuyns et al. (1987) carried out a case-control study involving 453 colonic and 365 rectal cancer cases and 2,851 population controls. Dietary fat was found not to be a significant risk factor for colon cancer. Rather, oligosaccharides were reported to be the important nutritional risk factor (Tuyns et al., 1987). More recent epidemiological studies have similarly observed a null association between dietary fat and colon cancer risk (Meyer and White, 1993; Peters et al., 1992).

Animal studies examining the effects of dietary fat on mammary and colon carcinogenesis have been stronger and more consistent than the epidemiological data. There is a general agreement that an increased intake of fat results in an increased incidence of mammary (Carroll and Khor, 1971; Kalamegham and Carroll, 1984; Tang et al., 1996) and colon tumors in animals (Reddy and Maeura, 1984; Reddy and Sugie, 1988;
Reddy, 1992). Carroll and Khor (1984) found that female Sprague-Dawley (SD) rats initiated with dimethylbenz[α]anthracene (DMBA), a mammary-specific carcinogen, and fed semi-synthetic diets containing 10% and 20% corn oil developed significantly more mammary tumors (~90%) than rats fed a low fat diet containing 0.5% or 5% corn oil (~70%). Reversal of the mammary-tumor promoting effects of a high fat diet (23%, w/w) has also been shown by lowering dietary fat levels to 5% by weight (Kalamegham and Carroll, 1984). As well, rats treated with DMBA and subsequently fed a diet lacking fat develop significantly fewer mammary carcinomas than control rats fed a diet with normal fat levels (5%, w/w) (Davidson and Carroll, 1982). Tang et al. (1996) found that when dietary levels of fat were increased from 15% to 30% of calories (or ~10-20%, w/w), mammary tumor incidence increased rapidly in MNU treated rats. However, mammary tumor incidence was not further influenced by dietary fat levels greater than 30% of total calories, suggesting that fat levels beyond this does not further promote mammary tumorigenesis (Tang et al., 1996).

A strong relationship between the amount of calories derived from dietary fat and colon cancer has also been reported in a variety of animal models. An early study by Nigro et al. (1975) studied the effect of diets containing 5% and 35% beef fat (w/w) on AOM-induced intestinal (small and large intestine) tumors in SD rats. Rats fed the high beef fat diet developed a significantly greater number of intestinal tumors and more metastases in the abdominal cavity, lungs, and liver, than animals fed the low beef fat diet. In a number of studies, Reddy et al. (1976, 1977, 1984, 1988) and others (Pence and Buddingh, 1988; Sakaguchi et al., 1984) have consistently shown that animals fed a high
fat diet containing 20% corn oil or lard (w/w) were more susceptible to AOM and
dimethylhydrazine (DMH)-induced colon tumors compared to rats fed a 5% corn oil or
lard diet (w/w). Diets high in dietary fat have also been shown to increase the number of
aberrant crypt foci (ACF), or putative preneoplastic lesions of colon cancer (Bird, 1987;
McClellan and Bird, 1988), in the AOM-induced rat model. ACF are putative precursors
of colon cancer, first described by Bird (1987), which give rise to colon cancer through a
multi-step process (Bruce et al., 1993). ACF represent the early stage of colon
carcinogenesis and serve as a good biomarker of colon cancer risk since they share similar
dysplastic characteristics and molecular abnormalities with colon tumors (Roncucci et al.,
1991; Stopera and Bird, 1992; Vivona et al., 1993). Lafave et al. (1994) have found that
a 15% beef tallow diet (w/w) promoted the growth of ACF more than a 5% beef tallow
diet (w/w) in SD rats treated with 20mg/kg AOM. Tang et al. (1996) and Shivapurkar et
al. (1996) have also demonstrated this promoting effect on ACF by a high fat diet (20%.
w/w) in SD rats treated with 30mg/kg AOM. In fact, linear regression analysis in both
these studies found that ACF determined after 11 weeks of experimental diet feeding
correlated with the final colon tumor incidence at 32 weeks (Shivapurkar et al., 1996;
Tang et al., 1996).

There is accumulating evidence to suggest that the type of dietary fat is an
important modulator of mammary and colon carcinogenesis in the rat. Diets high in
saturated and polyunsaturated fatty acids (i.e. ω-6 PUFAs), such as corn oil, have been
shown to enhance mammary (Carroll and Khor, 1971; Fay et al., 1997; Thompson et al.,
1989) and colon tumor incidence (Reddy, 1992; Reddy and Maeura, 1984; Reddy and
For instance, in a meta-analysis of 97 reports of experiments studying the effect on mammary tumor incidence of different types of dietary fatty acids, ω-6 PUFAs were shown to have a strong tumor-enhancing effect at different fat levels (Fay et al., 1997). However, when the intake of ω-6 PUFAs is at least 4% of calories, the ω-6 PUFA effect is stronger than that of saturated fats (Fay et al., 1997). Similarly, in a number of animal studies, Reddy and colleagues, have shown that a 24% corn oil diet (w/w) has a significantly greater promoting effect on colon tumors than diets containing various levels of menhaden (fish) oil, rich in ω-3 PUFAs (Reddy et al., 1991; Reddy and Sugie, 1988). Indeed, the ω-3 class of fats (found in fish, olive, perilla, and linseed oil) have been shown in many animals studies, in contrast to ω-6 PUFAs, to have a protective effect on both colon and breast cancer development (Fay et al., 1997; Karmali et al, 1984; Nelson et al., 1988; Reddy et al., 1991). However, the mechanism by which the type and amount of dietary fat modulates mammary and colon carcinogenesis is still unclear.

1.2.2.2 Energy Intake

In many of the epidemiological and animals studies where a positive association between dietary fat and breast and colon carcinogenesis was observed, the effect of energy intake was not considered. Since fat is the most energy-dense nutrient, high fat diets are generally also higher in calories. Consequently, it is difficult to separate the effects of fat and calories in both mammary and colon cancer development. In human populations, energy intake has been shown to be positively correlated with breast cancer mortality.
As well, many studies have shown that excessive body weight is directly associated with breast cancer risk, although only in post-menopausal women (Helmrich et al., 1983; Rose, 1986). A similar association between excess energy intake and colon cancer risk has been observed (Bostick et al., 1994; Giovannucci and Willet, 1994).

As discussed in Section 1.2.2.1, the possible promoting effect of a high fat diet on mammary and colon tumor development has been well studied (Fay et al., 1997; Reddy, 1992; Zhao et al., 1991). However, there has been controversy as to whether the tumor promoting effects of high fat diets are attributable to fat intake per se or to increased calories derived from a high fat diet (Carroll, 1986). In the rat mammary tumor model, Boissemault et al. (1986) have shown that tumor incidence and tumor growth rates are directly proportional to the net energy content of the diets. Furthermore, rats fed a low-fat/high-energy diet exhibited a higher number of mammary tumors than those fed an energy-restricted/high-fat diet (Boissemault et al., 1986; Kritchevsky, 1984). In a study by Klurfeld et al. (1989) designed to address the independent effects of total energy intake and dietary fat composition, a direct linear relationship between the degree of energy restriction (10%-40%) and the inhibition of DMBA-induced cancer promotion was reported. The diets used in this study were adjusted such that rats in the energy-restricted groups consumed comparable amounts of fat. Klurfeld et al. (1989) concluded that energy intake is a greater determinant than dietary fat on promoting mammary tumorigenesis. These results are in accord with a similar study aimed at examining the effect of energy intake on the promotion of mammary carcinogenesis by dietary fat in the MNU-induced mammary cancer model (Thompson et al., 1985).
The relationship between colon cancer risk and excess energy intake has not been well studied in the rat model. Clinton et al. (1992) reported that *ad libitum* feeding was significantly associated with intestinal (i.e. small and large intestine) carcinogenesis in SD rats. The effect of energy intake on intestinal carcinogenesis was greater than the effect of protein or fat level in this study (Clinton et al., 1992). Studies aimed at studying the effects of modulating energy expenditure on tumorigenesis have supported the possible promoting role of caloric intake in both mammary and colon tumorigenesis. Using the MNU-induced rat protocol, Cohen et al. (1981) found that mammary tumor incidence in rats fed a high fat diet (20% corn oil, w/w) decreased with voluntary exercise compared to control sedentary rats fed a low-fat diet. Similarly, Thorling et al. (1993) have shown that exercise training and a reduction in body weight significantly reduces colon tumor incidence in rats treated with AOM.

Studies on energy restriction provide further evidence for an important role of energy intake in breast and colon cancer development. A calorie-restricted diet has been consistently shown in the rat model to reduce the incidence and growth rate of mammary tumors (Klurfeld et al., 1987, 1989; Ross and Bras, 1971; Tannenbaum, 1945). A 40% calorific restriction in DMBA-initiated SD rats results in a 2-fold decrease in mammary tumor incidence as compared to *ad libitum* fed rats (Klurfeld et al., 1987). Indeed, a subsequent study by Klurfeld et al. (1989) found that the degree of tumor inhibition was dependent on the level of calorific restriction, such that 30% and 40% calorie-restricted diets inhibited mammary tumor development to a greater extent than a 20% calorific restriction. In AOM-initiated Fischer 344 (F344) rats, Kumar et al. (1990) reported that
a 20%-30% caloric restriction of a high fat 23% corn oil diet (w/w) reduced the incidence of colon tumors significantly to ~50% compared to a colon tumor incidence of 85% in ad libitum fed controls. This is in accord with results from Steinbach et al. (1993) who reported significant reduction in colonic cell proliferation, as determined by $[^3]$H]thymidine labelling, in AOM-initiated F344 rats calorically restricted 70%-80% of the kilocalories consumed by ad libitum fed rats. The striking evidence from animal studies supports an important role of energy intake on mammary and colon tumorigenesis. However, whether the enhancement of tumorigenesis by hyperalimentation with fat results from the metabolic activities of fat per se or excess calories from fat consumption is still controversial.

1.2.2.3 Carbohydrates

Epidemiological studies have suggested that sugar consumption is correlated with breast and colon cancer risk. Hems (1978) reported that a high intake of refined sugar was positively associated with an increased incidence of breast cancer in a survey of 41 countries. Similarly, Seely and Horrobin (1983) reported a connection between breast cancer incidence and sugar consumption, particularly in older women. In a case-control study of Canadian women, Lubin et al. (1981) found an increased frequency of consumption of sweet desserts by women with breast cancer. Franceschi et al. (1996) found that the amount of available carbohydrates had a greater impact than total fat intake on breast cancer risk in an Italian population. A similar relationship between sugar consumption and colon cancer incidence has also been reported in a number of studies (Centonze et al., 1993; La Vecchia et al., 1993; Tuyns et al., 1987). However, an inverse
relationship has been reported between dietary starch and breast cancer incidence, (Hems and Stuart, 1975) as well as colon cancer risk (Cassidy et al., 1994).

The digestibility of carbohydrates has also been shown to be a determining factor in the promotion or inhibition of colon and mammary cancer in the animal model. Diets high in simple carbohydrates have been shown to increase colonic cell proliferation and the growth of ACF (Caderni et al., 1991, 1993; Stamp et al., 1993). In contrast, diets high in starch, a low glycemic index carbohydrate, have been shown to be protective against colon carcinogenesis (Bianchini et al., 1992; Caderni et al., 1991, 1993; Thorup et al. 1995). Hoehn and Carroll (1979) also found that starch feeding (68% carbohydrate, w/w) resulted in decreased DMBA-induced mammary tumor incidence compared to simple sugars (i.e. sucrose or dextrose). However, Klurfeld et al. (1984) have reported no significant difference in mammary tumor incidence between rats fed a sucrose or corn starch-rich diet (i.e. 65%, w/w). The mechanism by which dietary carbohydrates can modulate tumorigenesis in the mammary gland and colon is still under investigation.

1.2.2.4 Insulin and Insulin Resistance

As discussed in Sections 1.2.2.1-1.2.2.3, evidence from epidemiological and animal studies have suggested that dietary factors including fat, energy, and carbohydrates are all involved in the etiology of both breast and colon cancer. Despite extensive study, the mechanism to explain the modulating effects of dietary factors on mammary and colon tumorigenesis is still unclear. However, it has been recognized that dietary fat, carbohydrate, and energy intake are all also risk factors associated with the development
of insulin resistance, impaired glucose tolerance, and hyperinsulinemia (Smith, 1994; Giovannucci, 1995) which are important in the syndrome of NIDDM. In light of this, hyperinsulinemia and insulin resistance have emerged as potential risk factors and modulators of mammary and colon cancer development.

Seely and Horrobin (1983) first proposed that a positive relationship between breast cancer rates and sugar consumption in international studies may be explained by insulin secretion. Several other epidemiological studies have also shown that refined sugars are a major dietary risk factor for colorectal cancer (Centonze et al., 1993; La Vecchia et al., 1993; Tuyns et al., 1987). Seely and Horrobin (1983) hypothesized that excessive stimulation of insulin release following high intake of sugars results in a hormonal environment that predisposes to mammary tumor development. Hyperinsulinemia is a compensatory response to the development of insulin resistance. Dietary factors which are associated with the development of insulin resistance are also those involved in breast cancer, such as fat and obesity/excess caloric intake. Therefore, Kazer (1995) and Stoll (1996) have proposed that insulin resistance may be a risk factor for breast cancer. A similar hypothesis suggesting that insulin and insulin resistance may be involved in colon cancer development has been proposed by McKeown-Eyssen (1994) and Giovannucci (1995). They have suggested that high risk diets and lifestyle factors lead to the development of insulin resistance and hyperinsulinemia, in which case insulin may act as a promoter of colon cancer. McKeown-Eyssen (1994) has further suggested that the elevated glucose and triglyceride levels associated with the development of
hyperinsulinemia and insulin resistance in NIDDM may provide a source of energy for the promotion of neoplastic cells and the development of colon cancer.

Several epidemiological studies have supported the hypothesis relating hyperinsulinemia and insulin resistance with the development of both breast and colon cancer. A case-control study of polyp patients and previous colon cancer patients by McKeowyn-Eyssen et al. (1996) has shown a positive association between colon cancer risk and fasting insulin, C-peptide, triglyceride levels, and central adiposity. Bruning et al. (1992) have also presented evidence that hyperinsulinemia with insulin resistance is a significant risk factor for breast cancer development in a case-control study comparing 223 women with operable breast cancer and 441 women of the same age without the cancer.

In many cases, hyperinsulinemia and insulin resistance precede the development of NIDDM (Alzaid, 1996). Thus, it would be anticipated that NIDDM would be associated with breast and colon cancer development. Interestingly, the few epidemiological studies that have been done have not supported such an association between breast cancer and NIDDM (Sellers et al., 1994; Kopp et al., 1990; Franceschi et al., 1990). In contrast, the small number of epidemiological studies that exist have shown a correlation between NIDDM and colon cancer risk (Adami et al., 1991; Weiderpass et al., 1997; Williams et al., 1984). For instance, Williams et al. (1984) analyzed case records from patients of two Mississippi hospitals and found a significantly higher frequency of overt diabetes mellitus among patients with colon cancer as compared to those with lung carcinoma or hip fractures.
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A potential role for insulin on breast and colon cancer development has also been evaluated in animal models. Animal studies have indeed supported an important effect of insulin on colon carcinogenesis. In the rat model, Tran et al. (1996) showed that exogenous insulin treatment promotes colon tumor development in rats initiated with the colon-specific carcinogen, AOM. As well, Corpet et al. (1997) have recently reported that exogenous insulin promotes the growth of ACF in rats also initiated with AOM.

Evidence to support insulin's role in mammary carcinogenesis has largely been indirect. Administration of insulin or glucose to tumor bearing rats significantly increased mammary tumor growth, an effect that was enhanced when the treatments were combined (Heuson et al., 1972; Heuson and Legros, 1970). In contrast, destruction of pancreatic β-cells by alloxan (Heuson and Legros, 1972) or streptozotocin (STZ) (Cohen and Hilf, 1975) in rats bearing mammary tumors resulted in significant tumor regression while insulin administration reactivated their growth. Heuson and Legros (1972) have also reported that the induction of alloxan diabetes after carcinogen administration completely prevented mammary tumor development. Their observation, however, may have been the result of considerable loss in body weight in the diabetic animals since caloric restriction has been known to inhibit rat mammary carcinogenesis (Welsch, 1992). In contrast to these results, a recent study by Lu et al. (1998) found that exogenous insulin does not promote MNU-induced mammary tumorigenesis using the protocol previously shown to promote colon tumorigenesis in AOM-initiated rats (Tran et al., 1996).

Insulin’s possible promoting effect on mammary and colon tumor development has also been suggested by a number of in vitro studies. Insulin has been shown to increase
cell proliferation and DNA synthesis in rat mammary carcinoma cells (Hallowes et al., 1977; Heuson et al., 1967; Heuson and Legros, 1971; Lewis and Hallowes, 1974; Osborne et al., 1976; Pasteels et al., 1976; Rudland et al., 1977; Welsch et al., 1976). Similarly, \textit{in vitro} studies have shown that insulin acts as a growth factor for normal colonic mucosal cells as well as a mitogen of colonic carcinoma cells (Koenuma et al., 1989; Wong and Holdaway, 1985).

The association of insulin and insulin resistance with colon and mammary tumorigenesis has also found support from indirect evidence suggesting that insulin is a common mediator of the promoting/inhibiting effects of dietary factors such as fat, energy, and carbohydrates. Animal studies have shown that high fat diets known to promote mammary (Dao and Chan, 1983; Goshal et al., 1994) and colon cancer (Reddy and Maeura, 1984; Reddy and Sugie, 1988) can also induce insulin resistance (Storlien et al., 1986). Furthermore, physical activity/exercise, which has been shown to reduce mammary (Cohen et al., 1981) and colon tumorigenesis (Thorling et al., 1993), is also known to improve insulin resistance (Nagasawa et al., 1995). In addition, caloric restriction is not only a strong inhibitor of breast (Klurfeld et al., 1995) and colon (Kumar et al., 1990) cancer but is also capable of improving insulin sensitivity, glucose uptake, and lowering insulin levels in rats (Escriva et al., 1992; Okauchi et al., 1995). Therefore, insulin with associated insulin resistance has emerged as a potential common mediator of the tumorigenic effects observed with various dietary factors associated with breast and colon cancer development. Consequently, insulin with associated insulin resistance has been hypothesized to be an important risk factor in the development of these two cancers.
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(Giovannucci, 1995; Kazer, 1995; Stoll, 1996; McKeowyn-Eyssen, 1994). However, the extent to which nutritional risk factors may modulate mammary and colon cancer promotion through insulin and insulin resistance has not been examined since an appropriate animal model has not yet been described.

1.2.3 The Obese (fa/fa) Zucker Rat - A Model to Study Breast and Colon Cancer

The Zucker (fa fa) rat or “fatty” rat is a genetically obese rat first described in 1961 by Zucker and Zucker (1961) (Figure 1.1). The most prominent metabolic abnormalities of this rat include hyperinsulinemia (Zucker and Antoniades, 1972), insulin resistance (Terretaz et al., 1986; York, 1972), glucose intolerance (Apweiler and Freund, 1993; Ionescu et al., 1985), hyperphagia (Bray and York, 1972), and hyperlipidemia (Barry and Bray, 1969; Zucker and Zucker, 1962). In contrast, lean Zucker rats (FA FA or FA fa) do not display these metabolic abnormalities. Traditionally, the obese Zucker rat has been used as a model of obesity and NIDDM. In light of the evidence to suggest that dietary factors (i.e. fat and carbohydrates), caloric intake, insulin and insulin resistance are risk factors for breast and colon cancer, the obese Zucker rat serves as a potential model to study the extent to which these factors can modulate cancer promotion. To fully understand and appreciate the potential use of the obese Zucker rat to study carcinogenesis, a detailed discussion of the various metabolic characteristics of the obese (fa:fa) Zucker rat follows.
Figure 1.1 The obese (fa/fa) and lean (FA/FA or FA/fa) Zucker rat.
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1.2.3.1 Phenotype and Genotype

The Zucker rat strain arose spontaneously from a cross between Sherman and Merck stock M rats (Zucker, 1961), representing a cross between a female albino and a true self-breeding black rat (Zucker, 1960). The obesity in fa/fa Zucker rats is inherited as an autosomal recessive mutation (Zucker and Zucker, 1961; Yen et al., 1977). Rats homozygous for this mutation exhibit various metabolic alterations in addition to obesity which are similar to the syndrome of NIDDM in humans, such as hyperinsulinemia, insulin resistance, and hyperlipidemia (Coleman, 1982).

Recently, Zhang et al. (1994) identified and characterized the obese (ob) gene that when mutated, causes hereditary obesity in the ob/ob mouse, a genetic model of obesity and diabetes. The ob gene encodes for leptin, a 16-KD peptide secreted by adipocytes. Leptin has been shown to act in the central nervous system to suppress appetite (Campfield et al., 1995). A mutation in the ob gene in these mice prevents leptin from exerting its satiety effect within the hypothalamus, resulting in hyperphagia, increased storage of fat, and obesity (Zhang, et al., 1994; Rink, 1994). In addition, animals show decreased physical activity, hypothermia, and infertility, suggesting that leptin may act to trigger an adaptation to starvation rather than acting as merely a satiety hormone (Ahima et al., 1996). Early experiments (Coleman, 1973; Coleman and Hummel, 1969) showed that another genetically obese/NIDDM mouse model, the db/db mouse, had functional leptin although satiety is not achieved. Tartaglia et al. (1995) and others independently traced the obesity seen in db/db mice to a mutation in the leptin receptor, OB-R (Chua et al., 1996; Tartaglia, 1995).
In 1991, Truett et al. (1991) reported that the defective \textit{db} gene in mice was homologous to the \textit{fa} gene of the genetically obese Zucker \textit{fa/fa} rats, although the \textit{db} gene maps to different chromosomes - mouse chromosome 4 and rat chromosome 5, respectively (Truett et al., 1995). Evidence that the \textit{fa} gene results in a defective hypothalamic \textit{OB-R} was first reported by Murakami and Shima (1995), who found that the expression level of \textit{ob} mRNA in the adipose tissue of Zucker rats is 4-fold higher than their lean littermates, suggesting that the \textit{ob} gene is functional. Iida et al. (1996a,b) have since cloned and sequenced two spliced variant forms of the rat \textit{OB-R} containing a short or long intracellular domain. They reported no differences in gene structure of the variant forms of the \textit{OB-R} in Zucker \textit{(fa/fa)} rats. Rather, Iida et al. (1996a) found a phenotype-linked nucleotide alteration in codon 269 of the extracellular domain in both variant forms of the Zucker \textit{(fa/fa)} \textit{OB-R} which results in a glutamine to proline amino acid substitution. This substitution at codon 269 of the leptin receptor is thought to be the crucial mutation which prevents leptin signalling from occurring in the hypothalamus and thus results in the obese phenotype of the Zucker \textit{(fa/fa)} rat (Iida et al., 1996b). Others have since reported a similar leptin receptor mutation in another genetically obese rat model, the Koletsky \textit{corpulent (cp)} rat (Kahle et al., 1997; Yen, 1977).

Leptin is thought to control food intake and body weight. However, the presence of variant forms of \textit{OB-R} derived from alternative splicing in other tissues, including the kidney, lung, and liver, has suggested that leptin may have other physiological functions (Tartaglia et al., 1995; Lee et al., 1996; Cioffi et al., 1996). Cohen et al. (1996) recently found an \textit{OB-R} variant in human hepatic cells. Exposure of these cells to leptin at
concentrations comparable with those found in obese individuals resulted in the attenuation of several insulin-induced activities. Specifically, tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), the association of the adapter molecule growth factor receptor-bound protein 2 (GRB2) with IRS-1, and down-regulation of gluconeogenesis (via the rate-limiting enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase, PEPCK) are attenuated (Cohen et al., 1996). Tyrosine phosphorylation of IRS-1 by the insulin receptor (IR) kinase is a key step in the insulin receptor signalling pathway and GRB2 mediates parts of this cascade (Cheatham and Kahn, 1996). The attenuation of insulin-induced signals in hepatic cells by leptin has provided evidence that leptin may antagonize some functions of insulin and thus may contribute to the development of obesity-associated insulin resistance, including that found in the obese Zucker rat.

The relationship between the ob gene and insulin resistance in the Zucker rat was first reported recently by Cusin et al. (1995). Cusin et al. (1995) showed that adipose tissue ob mRNA is upregulated by a rise in insulin levels in normal lean (F/A fa) Zucker rats as determined by the euglycemic clamp technique. The opposite effect was observed when these rats were fasted for three days to induce lower insulin levels. In adult obese (fa/ fa) rats, basal insulinemia and ob mRNA levels increase in parallel, such that ob mRNA levels are significantly higher than those of age-matched control lean rats. However, normalization of hyperinsulinemia induced by fasting failed to decrease the high ob mRNA levels in the obese rats (Cusin et al., 1995). The lack of downregulation of ob mRNA levels in response to changes in insulin levels of obese rats supports the idea that their
obesity and insulin resistance are due to both a defective OB-R and leptin's ability to affect the insulin receptor pathway.

1.2.3.2 Obesity

Rats homozygous for the fa mutation cannot be distinguished from normal lean rats until the third or fourth week of age, when the total body fat reaches approximately 20% of the body weight (Bell and Stern, 1977; Zucker and Zucker, 1961). Although detection of the obese phenotype can only be performed visually, the genotype can be determined at 16-18 days of age by various methods, including decreased oxygen consumption (Kaplan, 1979) and inguinal fat pad weight versus body weight (Lavau and Bazin, 1982). The faфа genotype may be identified as early as 7 weeks of age by the size and number of hypodermal adipocytes from skin biopsies (Hausman et al., 1983), by fat cell size from inguinal fat pad biopsies (Boulange et al., 1979), or by measuring core temperature of pups on two consecutive days (Schmidt et al., 1984).

From an early age, significant deviations in growth occur in both males and female faфа rats. Fat weight progressively accumulates throughout the life of the rat (Zucker and Antoniades, 1972). As with other genetically obese rodents, obesity in Zucker rats is a result of an energy imbalance. This is a consequence of both an increase in food intake and an elevation in the efficiency of food utilization, which reflects a decrease in energy expenditure. Hyperphagia has been found to account for some degree of the obesity in Zucker rats. Obese rats are known to consume more food than their lean littermates or rats that become obese as a result of an injury to the ventralmedial hypothalamic region of
the brain (i.e. VMH-lesioned rats) (Bray and York, 1972; Cnce et al., 1974; Zucker and Zucker, 1962). However, pair-feeding of obese Zucker rats to the food intake of lean rats does not normalize the body fat weight (Bray et al., 1973; Zucker, 1975). Even long-term food restriction in which obese rats received a reduction of 30% in food intake for 63 weeks did not affect retroperitoneal pad weight, hyperinsulinemia, hyperlipidemia, and serum albumin levels (Cleary et al., 1987). Rather, obese Zucker rats continued to weigh more than lean rats given the same food intake and yet the metabolic defects (e.g. hyperinsulinemia) in these obese rats were maintained (Bray et al., 1973; Cleary et al., 1980, 1987). In addition to hyperphagia, it seems that obese rats are also more efficient in their utilization of food, as evidenced by an increase in body fat per gram of food consumed (Bray 1970; Bray et al., 1973; Deb and Martin, 1976; Zucker, 1975).

The regulation of food intake of fa/fa rats has been well documented and has been compared to lean and VMH-lesioned obese rats. Obese Zucker rats eat larger and less frequent meals, although they tend to lose their nocturnal feeding patterns (Becker and Grinker, 1977). Food pattern intake in obese Zucker rats seems to be more similar to lean Zucker rats than to hypothalamic-obese rats. VMH-lesioned rats have been shown to decrease their food intake when the diet is diluted with cellulose/indigestible bulk (Bray and York, 1972; Cnce et al., 1974) whereas obese rats will adjust their caloric intake so as to maintain their body weight (Bray and York, 1972). Indeed, Greenwood et al. (1974) have noted a difference in food motivated behaviour between fa/fa rats and VMH-lesioned rats. Obese fa/fa rats were observed to increase their frequency of bar-pressing for food.
pellets as compared to VMH-lesioned rats, indicating a higher level of food intake (Greenwood et al., 1974).

With an increase in energy intake and storage in the *fa/fa* rat, excess body weight gained has been associated with an increased deposition of body fat and diminished protein deposition (Bray et al., 1973; Deb et al., 1976; Radcliffe and Webster, 1976, 1978). Thus, there is a clear partitioning of energy towards fat deposition in *fa/fa* rats. In addition, adult obese Zucker rats have a low level of spontaneous physical activity, contributing to their positive energy balance. Indeed, forced exercise has little effect on the obesity and energy balance in obese rats (Deb and Martin, 1975; Stern and Johnson, 1977).

The excess weight in *fa/fa* rats has been shown to be accommodated by an increase in adipocyte size (Bray, 1969) and number (Johnson et al., 1976; Stern and Greenwood, 1974) in the retroperitoneal and subcutaneous depots (Johnson et al., 1971; Lemmonier and Alexiu, 1974). The higher number of adipose cells can only be reduced partially but not prevented by dietary manipulation (Deb and Martin, 1975; Stern et al., 1972). Even dietary restriction prior to weaning does not prevent the increase in adipocyte number in the adipose tissue (Johnson et al., 1971). The obese Zucker rat differs from the VMH-lesioned obese rat where excess adipose weight is accommodated by hypertrophy of existing adipose cells (Hirsch and Han, 1969). In contrast, obese rats are capable of increasing the number of adipocytes in mid-adulthood when the adipocyte cell number in lean rats has plateaued (Johnson et al., 1976). However, the fatty acid composition of the triglycerides in the adipose tissue does not differ between obese Zucker and VMH-lesioned rats (Bray, 1969).
Although one of the other prominent features of the obese Zucker rat is its marked hyperinsulinemia, insulin can not account for the obesity in this animal. Stolz and Martin (1982) treated obese rats with streptozotocin (STZ) to eliminate endogenous insulin production and administered physiological doses of exogenous insulin to maintain normal glucose levels. Normalization of insulin levels in this rat only ameliorated the obesity partially. With the recent identification and characterization of a mutation in the leptin receptor of fa/ fa rats (Iida et al., 1996a,b), it seems that the obesity is predominantly caused by this hypothalamic defect. A non-functional form of the OB-R helps to explain the hyperphagia and the many sympathetic nervous system abnormalities found in the obese rat (i.e. thermoregulatory thermogenesis) (Ahima et al., 1996).

1.2.3.3 Hyperinsulinemia and Insulin Resistance

Insulin resistance and hyperinsulinemia have been well characterized in obese Zucker rats. An increase in serum insulin levels is detectable as early as 3-4 weeks of age, distinguishing them from their lean littermates (Zucker and Antoniades, 1972). In addition, insulin concentrations in the pancreas are also increased (Lemmonier et al., 1974). Indeed, insulin secretion from islet cells isolated from fa/ fa rats is enhanced in response to glucose (Schade and Eaton, 1975; Stern et al., 1972). Pancreatic islet tissue increases in size via hypertrophy and hyperplasia of the β-cells in response to increased insulin secretory demand in the obese rats (Shino et al., 1973; York et al., 1972). Koh et al. (1990) have shown that pancreatic preproinsulin mRNA and plasma insulin levels increase with age to a greater extent in obese than in lean Zucker rats, suggesting
enhanced insulin gene expression with increased fat deposition in \textit{fa} \textit{fa} rats. Despite the abnormal insulin levels, insulin from \textit{fa} \textit{fa} rats has been shown to have normal structure and biological potency (Laburthe et al., 1975). Caloric restriction by pair-feeding lowers, but fails to normalize, insulin levels of obese rats (Lemmonier et al., 1974; Stern et al., 1972; York and Bray, 1973). In fact, York and Bray (1973) have shown that dietary restriction to 75\% of that eaten by lean Zucker rats or total starvation (Zucker and Antoniades, 1972) is necessary for normal insulin levels to be restored in \textit{fa} \textit{fa} rats.

The development of hyperinsulinemia is associated with the onset of insulin resistance in certain tissues of obese rats. The \textit{fa} \textit{fa} rat develops hepatic and peripheral insulin resistance after weaning (Crettaz et al., 1980; Czech et al., 1978; Kemmer et al., 1979; Stern et al., 1972, 1975; Terretaz et al., 1986). However, insulin resistance seems to develop in muscle sooner than it does in white adipose tissue (Penicaud et al., 1987). Given the marked insulin resistance seen in \textit{fa} \textit{fa} rats, it is surprising that contradictory evidence exists regarding their basal glycemia and the glucose tolerance. Early studies reported normoglycemia (Herberg and Coleman, 1977; Zucker and Antoniades, 1972) and normal glucose tolerance in adult \textit{fa} \textit{fa} rats as tested by intravenous glucose administration (IVGTT) (Crettaz et al., 1980; Ionescu et al., 1985). However, it has since been shown using the oral glucose tolerance test (OGTT) that basal glycemia increases and glucose intolerance worsens with the development of diabetes in the obese rats (Ionescu et al., 1985).

The mechanism underlying the development of hyperinsulinemia and insulin resistance in \textit{fa} \textit{fa} rats is unclear. Skeletal muscle seems to be a primary site of insulin
resistance in obese Zucker rats (Crettaz et al., 1980; Kemmer et al., 1979; Smith and Czech, 1983). Haring et al. (1987) have shown that the insulin receptor (IR) is reduced in both skeletal muscle and in the liver by approximately 40%. Furthermore, Haring et al. (1987) have shown that the IR kinase in skeletal muscle has a decreased ability to phosphorylate substrates. Similarly, Slieker et al. (1990) have reported a decrease in autophosphorylation of the IR tyrosine kinase in the muscle of obese Zucker rats, but not in the liver. However, this defect in the IR is corrected with STZ treatment which eliminates the hyperinsulinemia in these obese animals (Slieker et al., 1990). In contrast, Van De Werve et al. (1987) have shown a decrease in insulin-stimulated protein kinase C activity, a post-receptor event, in the heart and liver tissue of obese Zucker rats. Therefore, these studies have suggested that insulin resistance and hyperinsulinemia involve both receptor and post-receptor defects. As discussed in Section 1.2.3.2, although insulin may play a role in the expression of the obese phenotype, hyperinsulinemia is not a necessary condition for the development of obesity in the obese Zucker rat.

Glucose transporters (GLUTs) which mediate glucose movement into muscle cells and adipocytes have been studied as potential mechanisms for insulin resistance in Zucker rats. The most prevalent glucose transporter in muscle and adipose tissue is GLUT-4. Friedman et al. (1988) have shown that GLUT-4 levels in the gastrocnemius muscles (i.e. leg muscle) of obese rats did not differ from lean Zucker rats. However, Sherman et al. (1988) and Zaninetti et al. (1989) have suggested that insulin resistance in fa/fa rats is the consequence of a defect in signal transduction, functional activity of the transporter,
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and/or translocation of the transporter rather than in the amount of transporter protein in the tissues.

1.2.3.4 Hyperlipidemia

One of the earliest abnormalities recognized in the obese Zucker rat is their hyperlipidemia (Barry and Bray, 1969; Zucker and Zucker, 1962). Cholesterol levels are elevated in fa/.fa rats, although significantly elevated triglyceride (TG) levels is the predominant abnormality (Barry and Bray, 1969; Witztum and Schonfeld, 1979; Zucker and Zucker, 1962). Furthermore, the concentration of all serum lipoprotein classes is increased in obese Zucker rats, particularly the very low density lipoprotein (VLDL) fraction (Schonfeld and Pfleger, 1971). The VLDL of obese rats contains a higher proportion of TGs than that of lean Zucker rats, although the lipoprotein composition is normal (Schonfeld and Pfleger, 1971; Schonfeld et al., 1974; Witztum and Schonfeld, 1979). The abnormalities in lipid levels of the obese Zucker rat resembles, in many respects, that seen in the syndrome of Type II diabetes or NIDDM in humans (Reaven, 1988; Steiner, 1994).

The pathogenesis of the lipoprotein abnormalities in obese Zucker rats is not entirely understood. Studies have shown that lipogenesis is increased in the liver of fa/ fa rats, but this is coupled with a concomitant decrease in fatty acid oxidation (Azain and Ontko, 1989; Godbole and York, 1978; Malewiak et al., 1985). As a result, hepatic production of TG-rich lipoproteins is increased in obese Zucker rats. However, Schonfeld et al. (1974) have also shown that activity of lipoprotein lipase (LPL), which hydrolyzes
plasma TGs, is higher in obese Zucker rats. This suggests that the production and removal rates for lipoproteins are enhanced in fa/fa rats. Therefore, the elevated TG levels in the obese Zucker rats are the consequence of an imbalance between the increased lipoprotein secretion and their removal into adipose storage, presumably in response to their hyperinsulinemia (De Gasqiet and Pequignot, 1973).

1.2.3.5 Reproductive Function

The female obese Zucker rat has reduced fertility (Saiduddin et al., 1973). There seems to be a delay in vaginal opening, prolonged estrus cycles, reduced uterine and ovarian weight, and absence of deciduomata formation in reserpine-induced pseudopregnancy (Saiduddin et al., 1973). These abnormalities are not corrected by restricting caloric intake to normal (Bray et al., 1973). Levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in fa/fa rats have been reported to be both similar (Saiduddin et al., 1973) and higher (Bray et al., 1973) than lean Zucker rats. This suggests that gonadotropin secretion and regulation in obese rats may be defective. However, lean and obese Zucker rats seem to have comparable levels of circulating estrogen levels, (Bivens and Olster, 1997) and the response of the uterus to exogenous estradiol is not impaired (Bray et al., 1976).

Reproductive function in male fa/fa rats have also been shown to be abnormal. Deb and Martin (1975) have reported decreased testicular size in male obese Zucker rats. Furthermore, male obese rats have a loss of preferential response to female pheromones.
Chapter One

(Hemmes and Vaid, 1977). These studies suggest an insufficiency in steroid secretion in male obese Zucker rats.

As a result of the diminished reproductive function in both male and female fa/fa rats, they are deemed to be ineffective breeders. Since the fa/fa trait is inherited as an recessive mutation, selective breeding of lean Zucker rats with normal reproductive function are used to derive the obese rats (Zucker and Zucker, 1961).

1.2.4 Chemically-induced Breast and Colon Cancer Animal Models

1.2.4.1 Chemically-induced Breast Cancer

The two most common experimental rat models used in the study of mammary carcinogenesis are those involving the chemical carcinogens 7,12-dimethylbenz(α)-anthracene (DMBA) or N-methyl-N-nitrosourea (MNU). There are several factors which make the DMBA and MNU protocols useful experimental systems to study breast cancer. Firstly, mammary tumors can be induced in high incidence with a short latency period and with little toxicity. Doses of 2.5 to 20mg DMBA can induce tumors with latencies of 2-6 months with a final tumor incidence of 100% (Russo and Russo, 1996b). MNU doses greater than 30mg/kg result in a final tumor incidence greater than 90% (McCormick et al., 1981; Thompson and Adlakha, 1991). Commonly, a single dose of 50mg/kg MNU is used in tumorigenesis studies as it results in a short tumor latency of 2-3 months with a final tumor incidence of 100% (McCormick et al., 1981). A single dose of either MNU or DMBA is sufficient to induce mammary tumors (McCormick et al., 1981) and when given
in these doses, no short term mortality and no changes in weight gain are observed (McCormick et al., 1981; Huggins et al., 1959).

Mammary tumor induction by DMBA and MNU is also dose-dependent. The incidence of mammary tumors and the number of tumors per tumor-bearing rat (i.e. tumor multiplicity) increase with DMBA or MNU dose while tumor latency is inversely related to carcinogen dose (McCormick et al., 1981; Thompson and Adlakha, 1991; Shimkin et al., 1969; Russo and Russo, 1996b). Since the majority of the tumors induced by DMBA and MNU are hormone responsive (Russo and Russo, 1996b), the susceptibility of the mammary gland and consequently the tumor incidence or multiplicity, are highly dependent on the age at the time of carcinogen administration. Maximal tumor incidence is observed when DMBA and MNU are given to young virgin females during the age of sexual maturity, approximately 45-60 days of age (Grubbs et al., 1986; Rose et al., 1980). This stage of mammary gland development is characterized by a high rate of proliferation of the glandular epithelium, a period when terminal end buds (TEB) are most actively differentiating into alveolar buds (AB) (Russo and Russo, 1978; Rose et al., 1980).

Although mammary carcinogenesis has been extensively studied for more than 30 years in the rat, chemical induction of mammary tumors in mice has been examined for more than 50 years (Russo and Russo, 1996a). DMBA, 3,4-benzyopyrene, 3-methylcholanthrene (MCA), 1,2,5,6-dibenzanthracene, and urethane have been used to induce mammary tumors in mice. In mice, mammary tumors develop in a relatively long period of time and multiple carcinogen doses are necessary to induce mammary carcinogenesis (Russo and Russo, 1996a). Furthermore, the hormoneResponsiveness of
chemically induced mammary tumors in mice has not been as thoroughly studied as in the rat (Russo and Russo et al., 1990). Therefore, chemically-induced rat models of breast cancer are more useful for examining factors involved in the modulation of breast cancer development.

Despite the similarities in the DMBA and MNU models of chemically-induced mammary carcinogenesis, DMBA and MNU are different in their modes of initiation. DMBA requires metabolic activation by aryl hydrocarbon hydroxylase in mammary cells (Chan and Dao, 1983) whereas MNU is a direct-acting carcinogen (Gullino et al., 1975). Metabolism of DMBA to polar intermediates, including epoxides, are thought to be responsible for causing DNA damage in the initiation of carcinogenesis (Russo et al., 1990). In contrast, MNU is not dependent on metabolic enzymes to initiate carcinogenesis. Rather, the methyldiazonium ion formed from the chemical decomposition of MNU at physiological pH is the ultimate carcinogen responsible for the methylation of DNA in the initiation of carcinogenesis (Gullino et al., 1975). Since MNU is direct acting, its carcinogenic potency is not dependent on the metabolic enzymes of the animal which may differ between species and strains.

Although DMBA and MNU are known to induce mammary carcinogenesis in rats, different rat strains show various levels of susceptibility to these carcinogens. Sprague-Dawley (SD) and Wistar-Further (WF) rats are the most susceptible strains to DMBA and MNU-induced mammary carcinogenesis (Russo and Russo, 1996b). Fischer 344 (F344) and ACI rats exhibit intermediate susceptibility while Copenhagen rats are essentially completely resistant to mammary carcinogenesis (Russo and Russo, 1996b). The
similarity in response to DMBA and MNU-induced mammary cancer in different rat strains suggest that similar genetic factors control the level of susceptibility. However, the genetic mutations identified in MNU and DMBA-induced mammary tumors are different. It has been reported that 80-90% of MNU-induced mammary tumors contain \( c-Ha-ras \) mutations (Zarbl et al., 1985; Sukumar et al., 1983). In contrast, DMBA-induced mammary tumors resemble human breast cancer in that tumors rarely contain \( ras \) gene mutations (Rochiltz et al., 1989). An overexpression of the 'normal' \( c-Ha-ras \) oncogene product, p21, has been shown in both DMBA-induced and human mammary tumors (Clair et al., 1982; DeBartoli et al., 1985; DiBiasi et al., 1989; Thor et al., 1986). Despite differences in the molecular basis for the initiation of DMBA and MNU-induced mammary carcinogenesis, the pathogenesis of chemically-induced rat and human breast cancer is similar (Russo et al., 1990).

1.2.4.2 Chemically-induced Colon Cancer

The two most widely used colon cancer carcinogens in the rat model are 1,2-dimethylhydrazine (DMH) and its metabolite, azoxymethane (AOM). DMH is a procarcinogen, requiring a series of chemical transformations \textit{in vivo}, primarily in the liver, to form the ultimate carcinogen (Fiala, 1977). Briefly, DMH is metabolized to produce azomethane (AM) in the liver which is further metabolized to azoxymethane (AOM). AOM is subsequently converted to methylazoxymethanol (MAM). Cytochrome P\textsubscript{450} 2E1 (CYP2E1) is responsible for the activation of AOM and its proximate metabolite MAM (Sohn et al., 1991; Sohn and Fiala, 1995). The specific enzymes involved in the other
steps of DMH and AOM metabolism have not been completely characterized (Fiala et al., 1987). Upon the conversion of AOM to MAM, MAM is transported via the bloodstream to the colon where it breaks down under physiological conditions to form formaldehyde and methyldiazonium ion. The methyldiazonium ion is a highly reactive species which is responsible for the methylation of DNA and other macromolecules in the initiation of carcinogenesis (Fiala, 1977). Figure 1.2 summarizes the *in vivo* steps involved in the metabolism of DMH and AOM.

AOM and DMH have been used to study colon cancer development in both rats and mice. The advantage of these carcinogens is their ability to induce a high colon tumor incidence in a relatively short period of time. Ward (1975) has demonstrated that AOM can induce colon tumors in rats in a dose-dependent fashion. A single dose of 30mg/kg of AOM results in an 80% colon tumor incidence 48 weeks post-AOM (Ward, 1975). Similar tumor incidences and latencies are observed with two doses of 15mg/kg AOM (Holt et al., 1996; Reddy and Tanaka, 1986). However, higher colon tumor incidences (~100%) with a shorter latency period are observed with multiple weekly injections of DMH and AOM in rodents (Klurfeld, 1995; Ward, 1975).

Although DMH and AOM are known inducers of colon carcinogenesis in all rodents, they are most effective in rats (Nigro, 1985). Various rat strains, however, differ in their susceptibility to DMH or AOM-induced colon carcinogenesis. Sprague-Dawley and Long-Evans (LE) rats have been shown to be most susceptible, Buffalo and Wistar-Furth rats are intermediate in susceptibility, and Lobund-Wistar rats are resistant to DMH-
Figure 1.2 In vivo metabolism of 1,2-dimethylhydrazine (DMH) and azoxymethane (AOM). Adapted from Fiala, 1977 and Fiala et al., 1987.
induced colon carcinogenesis (Takizawa et al., 1978; Asano and Pollard, 1978). Differences in susceptibility to DMH-induced cancer have also been observed in various mouse strains (Evans et al., 1974, 1977). The genetic factors responsible for the susceptibility of rodents to DMH and AOM have not yet been determined.

AOM and DMH-induced colon carcinogenesis in rodents have been shown to share similar pathological features to that observed in the human disease. Both carcinogens have been shown previously to induce the development of ACF, which are putative preneoplastic lesions (Bird, 1987; McLellan and Bird, 1988). As discussed briefly in Section 1.2.2.1, ACF represent an early stage in the multi-step process of colon carcinogenesis (Archer et al., 1992; Bruce et al., 1993). Initiation of colonic cells from exposure to DMH or AOM result in abnormal growth of these cells which accumulate to form an aberrant crypt (AC). ACs are distinguished by their increased size, thicker epithelial lining, and increased pericryptal zone (Bird, 1987; McLellan and Bird, 1988). ACs further develop into ACF as many aberrant crypts cluster together. ACF can then proceed to form adenomas and adenocarcinomas which can acquire the characteristics observed in malignant tumors (Bruce et al., 1993). The dysplastic characteristics and molecular abnormalities associated with ACF are similar to those found in colonic tumors of both humans and AOM and DMH-initiated rats, suggesting that ACF are indeed preneoplastic lesions of colon cancer (Roncucci et al., 1991; Stopera and Bird, 1992; Vivona et al., 1993). For instance, it has been shown that 30-70% of DMH- or AOM-induced ACF in the rat colon contain Ki-ras mutations (Jacoby et al., 1991; Llor et al., 1991; Vivona et al., 1993), similar to that found in human colonic tumors (Vogelstein et
Therefore, the ACF-adenoma-carcinoma sequence of colon carcinogenesis observed in AOM and DMH-initiated rats has made it possible to study the factors involved in the initiation and promotion of colon cancer in an animal model.

1.2.4.3 Dual Organ Model for Breast and Colon Cancer

Recently, Shivapurkar et al. (1996) described a dual organ rat carcinogenesis bioassay for breast and colon cancer. Unlike conventional bioassays which use only one target organ as the endpoint, a dual organ model is rapid, reliable, and economical. Although there have been previous reports of multiple target organ carcinogenesis models, the individual carcinogen treatments in these studies were not specific for a single target organ and the objective of these studies was only to induce neoplasia at different sites in the same animal (Hirose et al., 1990, 1991; Shibata et al., 1990). Therefore, it was not clear in these studies whether there was an interaction effect from sequential exposure to more than one carcinogen. Shivapurkar et al. (1996) have demonstrated that colon and mammary tumor incidences are comparable in rats treated with both MNU and AOM either independently or in combination, suggesting the absence of an interacting effect between carcinogens.

Clearly the dual organ model is useful for comparing the potential chemopreventative or promoting effects of various agents on mammary and colon tumorigenesis. Using the dual organ model, a high fat diet (20%, w/w) has been shown to promote both mammary and colon cancer in rats (Shivapurkar et al., 1996; Tang et al.,
1996) while phytic acid (PA) supplementation in a high fat diet has been shown to reduce this promoting effect (Shivapurkar et al., 1996). In these studies, colonic ACFs and mammary tumors assayed at earlier time points have been shown to correlate with the final colon tumor incidence. Thus, the dual organ protocol can also be used to evaluate factors that modulate the early steps of colon and mammary carcinogenesis. Furthermore, the dual organ bioassay provides a model to study the interaction between chemopreventative and promoting agents with the molecular genetics of both colon and mammary cancer. As discussed in Section 1.2.4.1 and 1.2.4.2, Ki-ras mutations and Ha-ras mutations have been identified as important genetic alterations in the development of colon cancer and breast cancer, respectively, in the rat (Sukumar et al., 1983; Zarbl et al., 1985; Shivapurkar et al., 1994; Kumar et al., 1990a). Therefore, environment-gene interactions can be studied for both cancers simultaneously in a single model. The use of the dual organ protocol for chemically-induced mammary and colon cancer in the obese (fa fa) Zucker rat, hence, serves as a potential model to study the mechanism(s) by which dietary factors can modulate both breast and colon cancer development.
1.3 **Research Hypothesis and Objectives**

Evidence from epidemiological studies, animal and *in vitro* experiments have suggested that various dietary factors modulate mammary and colon carcinogenesis. The *fa fa* Zucker rat is genetically predisposed to developing obesity which is accompanied by a number of metabolic abnormalities characteristic of NIDDM, such as hyperinsulinemia, insulin resistance, and hyperphagia. Therefore, the obese (*fa fa*) Zucker rat serves as a potential model for studying whether these metabolic abnormalities play a role in mammary and colon cancer development. If the Zucker rat strain is indeed susceptible to both cancers, the hypothesis addressed in this thesis is that obese (*fa fa*) Zucker rats are more susceptible towards the development of breast and colon cancer than lean (*FA FA* or *FA fa*) Zucker rats. The metabolic abnormalities that arise from the genetic background of obese Zucker rats may act, individually or in combination, to increase their susceptibility to mammary gland and colon carcinogenesis as compared to lean Zucker rats.

The two main objectives are as follows:

1. To determine the susceptibility of the Zucker rat strain, including both lean (*FA FA* or *FA fa*) and obese (*fa fa*) rats, towards both breast and colon cancer using a dual organ rodent model of carcinogenesis in which cancers are initiated with a mammary-specific carcinogen, MNU and a colon-specific carcinogen, AOM.

2. To determine the relative susceptibility of obese and lean Zucker rats towards mammary gland and colon carcinogenesis.
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Materials and Methods
Chapter Two

2. Materials and Methods

2.1 Experimental Animals

Female lean (FA/FA or FA:fa) and obese (fa/fa) Zucker rats (5 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Growth curves were used by Charles River Laboratories (Wilmington, MA) to distinguish between lean and obese Zucker rats. The animals were housed in plastic cages with wire tops at 22 ± 2°C at 50% humidity and with a 12-hour light-dark cycle. Tap water and a pelleted standard AIN-93M diet (Dyets, Bethlehem, PA) were provided ad libitum throughout the experiment. The composition of the AIN-93M diet is presented in Table 2.1.

2.2 Preparation of Carcinogens

2.2.1 N-methyl-N-nitrosourea (MNU)

MNU (Sigma Chemical CO., St. Louis, MO) was dissolved in 0.05% (v/v) acetic acid in normal saline (0.9% NaCl, w/v) and administered intraperitoneally (i.p.) to the animals using a 27-gauge needle within 30 minutes of preparation.

2.2.2 Azoxymethane (AOM)

AOM (Sigma Chemical Co., St. Louis, MO) was dissolved in normal saline (0.9% NaCl, w/v) and immediately administered i.p. using a 27-gauge needle.
Chapter Two

Table 2.1 - Percentage by weight composition of the AIN-93M diet

<table>
<thead>
<tr>
<th>Composition</th>
<th>% by Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>46.6</td>
</tr>
<tr>
<td>Casein</td>
<td>14.0</td>
</tr>
<tr>
<td>Dextrinized Cornstarch</td>
<td>15.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>4.0</td>
</tr>
<tr>
<td>Alphacel, non-nutritive bulk</td>
<td>5.0</td>
</tr>
<tr>
<td>AIN-93-MX</td>
<td>3.5</td>
</tr>
<tr>
<td>AIN-93-VX</td>
<td>1.0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0.18</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.25</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.0008</td>
</tr>
</tbody>
</table>
2.3 Mammary and Colon Tumorigenesis Experimental Protocol

30 lean (FA/FA or FA:fa) and 30 obese (fa:fa) Zucker rats were acclimatized for two weeks. At 50 days of age, all animals received an i.p. injection of 37.5mg/kg MNU. On weeks one and two post-MNU (57 and 64 days of age), the animals received an i.p. injection of 15mg/kg AOM. These doses correspond to approximately 6.7-10mg MNU and 3.0-4.6mg AOM/obese rat, and 4.3-6.1mg MNU and 1.9-2.5mg AOM/lean rat. Body weights were recorded weekly. Blood samples were collected every 6 weeks from rats randomly chosen from each group. Blood was collected from the animals in the morning (~9:30am) under fed-state and fasting (i.e. 4 hours) conditions for serum insulin, glucose, and TG analysis. The experimental protocol is summarized in Figure 2.1.

The animals were palpated for mammary tumors 4 weeks following MNU treatment and weekly thereafter. The location of each mammary tumor was recorded. Rats with mammary tumors ≥ 20mm in diameter or exhibited rectal bleeding due to colonic tumors were sacrificed immediately. Halothane (Sigma Chemical Co., St. Louis, MO) inhalation followed by cervical dislocation was used to sacrifice animals. Rats remaining 20 weeks post-MNU were sacrificed and mammary tumors were collected for both storage at -70°C and fixation in 10% buffered formalin. Colons were also excised, flushed with phosphate-buffered saline (PBS, 10mM, pH 7.2), slit open and fixed flat in 10% buffered formalin between two layers of filter paper to assess the presence of tumors (approximately >2mm in diameter).
Figure 2.1  Experimental Protocol for Mammary and Colon Tumorigenesis
Animals were fed AIN-93M throughout experiment.  n = 30/group.
Blood samples were taken every 6 weeks for glucose, insulin, and triglyceride analysis.
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2.4 DNA Methylation Dosimetry

2.4.1 Biologically Effective MNU Doses in Zucker Rats

5 week old lean (FA:FA or FA:fa) and obese (fa:fa) Zucker rats were acclimatized for 2 weeks. To determine the biologically effective carcinogen dose, the rats were administered various doses of MNU for the analysis of 7-meG levels in mammary epithelial cells. At 50 days of age, lean rats (5/group) were treated with an i.p. injection of either 25 or 37.5 mg/kg MNU. Obese rats at 50 days of age (5/group) received an i.p. injection of either 20, 25, or 37.5 mg/kg MNU. Animals were sacrificed by CO2 asphyxiation and cervical dislocation. Rats were sacrificed 6 hours post-MNU for the analysis of 7-meG levels since DNA alkylation is near-maximal at that time (Herron and Shank, 1981; Cox and Irvine, 1979; Lu et al., 1992). At the time of sacrifice, mammary glands from the MNU-treated rats were excised, washed in cold PBS, and used immediately for epithelial cell isolation. Subsequently, epithelial cells were used for DNA extraction and 7-meG analysis by high performance liquid chromatography (HPLC). The experimental protocol to determine the biologically effective MNU doses in Zucker rats is summarized in Figure 2.2.

2.4.2 Biologically Effective AOM Doses in Zucker Rats

Lean (FA:FA or FA:fa) and obese (fa:fa) Zucker rats were acclimatized for 3 weeks upon their arrival. At 57 days of age, lean and obese rats (5/group) received an i.p. injection of 15mg/kg AOM. Animals were sacrificed by CO2 asphyxiation and cervical
Figure 2.2 Experimental Protocol for Measuring Biologically Effective MNU Doses

Animals were fed AIN-93M throughout experiment.
Animals were sacrificed 6-hours post-MNU treatment. n = 5 rats/group.
dislocation. Again, animals were sacrificed 6 hours post-carcinogen treatment for 7-meG analysis as DNA alkylation is near-maximal (Herron and Shank, 1981; Cox and Irvine, 1979; Lu et al., 1992). Colons of AOM-treated rats were rapidly removed, rinsed in cold normal saline (0.9% NaCl, w/v) and slit longitudinally. The colonic muscosa was scraped with a microscope slide to collect colonic epithelial cells. DNA was subsequently extracted from colonic epithelial cells for HPLC analysis for 7-meG levels. The experimental protocol to determine the biologically effective AOM doses administered to Zucker rats is summarized in Figure 2.3.

2.5 Assays of Serum Glucose, Insulin, and Triglyceride Levels

Following the two week acclimatization period in the tumorigenesis study, blood samples were collected every 6 weeks for glucose and insulin analysis in 12-16 rats randomly selected from each group. Blood samples were collected in the morning (~9:30am) from animals in the fed-state or after a 4 hour fast using the retro-orbital sinus technique. For serum TG levels, fed-state blood samples were taken from 16 rats/group randomly chosen at 7 and 11 weeks of age. Serum was prepared from the blood samples by centrifugation at 1500 x g for 15 minutes at 4°C in an IEC MicroMax centrifuge.

2.5.1 Serum Glucose Analysis

Serum glucose levels were measured enzymatically (Sigma Chemical Co., St. Louis, MO, Cat #510) based on the Raabo and Terkiilsen procedure (1960) which
Lean or Obese Zucker rats

AOM 15mg/kg i.p.

- colonic epithelial cells isolated from tissues
- DNA isolated from cells
- HPLC analysis of DNA samples for quantitation of 7-meG levels

Age (weeks)

Figure 2.3  Experimental Protocol for Measuring the Biologically Effective AOM Dose

Animals were fed AIN-93M throughout experiment.

Animals were sacrificed 6-hours post-AOM treatment. n = 5 rats/group.
involves the simultaneous use of glucose oxidase and peroxidase coupled with a chromogenic oxygen acceptor (o-dianisidine). 25μL of serum sample or a Standard glucose solution (100mg/dL) was added to 0.5mL of water and 5.0 mL of a combined mixture containing glucose oxidase, peroxidase, and o-dianisidine. A Blank solution containing 0.5mL water and 5.0mL of the combined enzyme mixture was used as a reference. The reaction was allowed to proceed to completion in 30 minutes at 37°C or 45 minutes at 18-26°C. The intensity of the brown color measured at 450nm is proportional to the original glucose concentration. The glucose levels (mg/dL) were calculated as A_sample/A_standard x 100.

2.5.2 Serum Insulin Analysis

Serum insulin was measured by radioimmunoassay (RIA) (ICN Pharmaceuticals, Costa Mesa, CA, Cat #06-D1804). The principle of the RIA involves the competitive binding of the serum insulin sample with an added known amount of 125I-labelled insulin for the binding sites on a specific antibody. With the addition of higher amounts of nonradioactive insulin, less radioactive insulin remains bound to the antibody until an equilibrium between the free and antibody-bound insulin occurs. Specifically, 100μL of insulin Standards, insulin Controls (30 and 90μIU/mL) or serum samples was added to 900μL of 125I-insulin dissolved in PBS, pH 7.4. The antibody is covalently bound to the inner surface of a polypropylene tube, resulting in an antibody-bound insulin complex which is also attached to the tube wall. Following an overnight (~18hr) incubation at
room temperature, the free antigen is aspirated or decanted, leaving only the antibody-bound insulin. Each tube was rinsed twice with deionized water and counted on a gamma counter calibrated for $^{125}$I to determine the amount of antibody-bound $^{125}$I-insulin. Concentrations of insulin in the serum samples were determined graphically from a standard curve obtained from the results of insulin standards (ICN Pharmaceuticals, Costa Mesa, CA, Cat #06-D1804). The measured radioactivity is inversely proportional to the amount of insulin present in the serum sample.

2.5.3 Serum Triglyceride Analysis

Serum TG levels were analyzed using a modified enzymatic method (Sigma, Chemical Co., St. Louis, MO, Kit #339) described by McGowan et al. (1983). Briefly, 10μL of deionized water (Blank), glycerol Standard (250mg/dL), lipid Control (350mg/dL), or serum samples was added to 1.0mL of a mixture (GPO-Trinder reagent) containing lipoprotein lipase (LPL), adenosine triphosphate (ATP), glycerol kinase (GK), glycerol phosphate oxidase (GPO), 4-aminoantipyrine (4-AAP), and sodium $N$-ethyl-$N$-(3-sulfopropyl)m-anisidine (ESPA). Initially, the triglycerides (TG) in the sample are hydrolyzed to glycerol and free fatty acids (FFAs) by LPL. The resulting glycerol product from TG hydrolysis by LPL undergoes a coupled reaction catalyzed by GK and GPO. The hydrogen peroxide ($H_2O_2$) formed then reacts with 4-AAP and ESPA to produce a quinonemine dye which shows an absorbance maximum at 540nm. Thus, the increase in absorbance at 540nm is directly proportional to the concentration of triglycerides in the
sample. Triglyceride levels (mg/dL) were calculated by \[\frac{A_{\text{test}} - A_{\text{blank}}}{A_{\text{standard}} - A_{\text{blank}}} \times \]
concentration of Standard.

2.6 Determination of Estrus Cycle Length

To determine if sex hormones affected the results of the tumorigenesis experiment, the estrus cycle length was measured in 10 animals/group at 23 weeks of age. For two weeks at approximately 10:00am, vaginal smears were collected by using a saline-moistened swab stick tip which was carefully inserted in the vagina and slowly rotated to collect a vaginal sample. The vaginal smear sample was transferred to a microscope slide and stained with Camco Quick Stain II (VWR Canlab, Mississauga, ON, Cat #71130-004). Stained slides were viewed under a light microscope at 40X to examine the cell types present. Vaginal changes in the rat are related to the estrus cycle. The criteria, as described by Baker (1979), used to determine the specific estrus cycle stage by vaginal smears is listed in Table 2.2. The average number of days of the estrus cycle for each rat was determined after two weeks of vaginal smear sampling (approximately 3 cycles).

2.7 Preparation of Mammary and Colonic Epithelial Cells

Mammary and colonic epithelial cells were isolated from their respective tissue as these are the target cells of the carcinogens MNU and AOM. A representative sample of colonic epithelial cells was collected by carefully scraping the luminal side of the colon (i.e. colonic mucosa) with a microscope slide. Colonic epithelial cells were washed
Table 2.2 - Criteria for the Interpretation of Vaginal Smears

<table>
<thead>
<tr>
<th>Estrus Cycle Stage</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-estrus</td>
<td>mainly leucocytes present, with or without mucous</td>
</tr>
<tr>
<td>Pro-estrus</td>
<td>mainly round epithelial cells</td>
</tr>
<tr>
<td>Early estrus</td>
<td>mainly cornified epithelial cells present</td>
</tr>
<tr>
<td>Late estrus</td>
<td>large numbers of adhering cornified cells</td>
</tr>
<tr>
<td>Met-estrus</td>
<td>both cornified cells and leucocytes present in large numbers</td>
</tr>
</tbody>
</table>
and stored in cold PBS. Mammary epithelial cells were purified from the mammary gland as described by Fong et al. (1990) and Lu et al. (1992), with modifications. Briefly, mammary glands were cut into small pieces (~1mm²) with scissors and/or a scalpel. Minced mammary glands were subsequently digested with Type III collagenase (Worthington Biochemicals, Freehold, NJ) in PBS at a concentration of 750 U/g tissue for 2 hr at 37°C with shaking. The digest was then centrifuged at 1000 r.p.m. for 5 minutes and the upper lipid layer and fat cells were removed by aspiration. The cell pellet was washed twice with PBS. Epithelial cells were collected by centrifugation and stored in PBS. This method of purifying epithelial cells from the mammary gland has been previously shown to result in preparations containing predominantly mammary ductal cells (Fong et al., 1990).

2.8 DNA Isolation

DNA from mammary and colonic epithelial cells was isolated using a modified phenol extraction method (Kirby, 1956). Firstly, cells were lysed by manual homogenization with a mortar and pestle and suspended in a digestion buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA. Addition of the digestion buffer to the minced cells protects the DNA from the action of nucleases throughout the protocol. Deproteinization was achieved by the addition of 20% SDS and 10 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) [final concentration of 0.5% SDS and 0.2 μg/mL, respectively] to the cell suspension which was then incubated overnight at
DNA was isolated with successive extractions using sevag (chloroform:isoamyl alcohol, 24:1), Tris-buffered phenol (1M Tris-HCl, pH 7.5, saturated), and a mixture of sevag/phenol (50:50). The phenol method is the most commonly used method for deproteinizing DNA which efficiently denatures proteins and dissolves denatured protein (Kirby, 1956). Chloroform is not only a protein denaturant but also serves to stabilize the interface between an aqueous phase and a pure phenol layer. The phenol/chloroform mixture reduces the amount of aqueous solution retained in the organic phase, maximizing the yield of DNA (Palmiter, 1974; Penman, 1966). Denatured protein forms an interface layer between the aqueous and organic phases. Through successive extractions, DNA contained in the aqueous layer was isolated. DNA was precipitated from the aqueous layer by adding 2 volumes of isopropanol at room temperature and recovered by centrifugation for 2 minutes at 1700 x g. The DNA was washed with 100% cold ethanol (kept at -20°C). The ethanol was decanted and the DNA pellet was allowed to air dry. The DNA was resuspended in Tris-EDTA (TE) buffer until dissolved. The purity and concentration of the DNA were determined spectrophotometrically. Highly pure DNA has an $A_{260}/A_{280}$ ratio $> 1.8$. As well, the DNA concentration was calculated using an $A_{260}$ of 20 = 1mg/ml (Current Protocols in Molecular Biology, 1994).
2.9 HPLC and 7-Methylguanine Measurement

The removal of 7-meG from DNA by cleavage of the N-glycosidic bond was achieved by neutral thermal hydrolysis. Briefly, DNA was heated at 100°C for 30 minutes in 5mM potassium phosphate buffer, pH 7.4. In contrast to deoxyguanosine, the glycosidic bond of 7-methyl deoxyguanosine is unstable and breaks easily on heating at neutral pH. Thus, neutral thermal hydrolysis selectively releases 7-meG from DNA (Kriek and Emmelot, 1964; Lawley and Brooks, 1963). Park and Ames (1988a, 1988b) have shown that 7-meG in DNA is consistently released quantitatively (>97%), chromatographed as a single major peak on HPLC.

The hydrolysate was analyzed by reverse-phase HPLC using an Ultrasphere ODS column (Beckman, 4.6mm x 250mm, 5μm diameter). The mobile phase consisted of a 5mM potassium phosphate buffer (pH 5.5) with 2% methanol (Park and Ames, 1988a, 1988b). A Waters 490 Programmable multiwavelength detector (Waters, Millford, MA) was used for detection at 260nm. The concentrations of 7-meG in the hydrolysate samples were determined by comparing peak areas to standard solutions of authentic 7-meG (Sigma Chemical Co, St. Louis, MO). The levels of DNA methylation are expressed as mmol 7-meG/mol DNA, assuming 1ug DNA = 3240 pmol of deoxynucleoside monophosphates (Schaffer et al., 1996).
2.10 Statistical Analysis

Values for body weights, serum insulin, glucose, triglycerides, tumor multiplicity, and estrus cycle length are reported as means ± SEM, with the number of animals/group indicated. Differences between the two groups for these parameters were determined using a Student’s t-test. Final cumulative mammary and colon tumor incidences are shown as a percentage and analyzed by the χ²-test. The rate of mammary tumor appearance was analyzed by the Mantel-Haenszel procedure (Mantel, 1966).

Values for DNA methylation (mmol 7-meG/mol DNA) are reported as means ± SEM, with the number of animals/group indicated. Differences between the groups were determined using a Student’s t-test. P<0.05 was taken to indicate statistically significant differences in all statistical analyses.
CHAPTER THREE

Experimental Results
3. Experimental Results

3.1 Mammary and Colon Tumorigenesis in Zucker Rats

3.1.1 Body Weights

The body weights of the obese rats were significantly higher than those of the lean rats at all ages throughout the tumorigenesis experiment. The final body weight of the obese rats was 526.9 ± 7.3g, which was significantly greater (P<0.0001) than the 265.4 ± 4.2g of the leans. Figure 3.1 shows the growth curve of both obese and lean rats throughout the experiment.

3.1.2 Serum Glucose, Insulin, and Triglyceride Levels

Obese Zucker rats are a well characterized model of NIDDM and obesity (Bray, 1977; Coleman, 1982). To monitor the development of insulin resistance, hyperinsulinemia, and hyperlipidemia, serum insulin, glucose, and triglyceride levels were measured.

3.1.2.1 Serum Glucose Levels

Fed-state serum glucose levels of 7, 13, 19, 25 week old lean and obese rats and fasted levels of 19 and 25 week old animals are shown in Figure 3.2. Fed-state glucose levels were significantly higher in the obese rats compared to the leans both at 13 (173.4 ± 6.6 versus 156.1 ± 5.0 mg/dL, respectively, P<0.05) and 25 weeks of age (187.6 ± 11.4 versus 146.4 ± 6.9 mg/dL, respectively, P<0.05). Although the obese Zucker rat is known
Figure 3.1  Weekly body weights (mean ± SEM) of ■ lean and ● obese Zucker rats. n = 30 rats/group. Body weights of obese rats are significantly different from the lean group at all ages.
Figure 3.2  Serum glucose levels (mg/dL) of lean (white bars) and obese (black bars) Zucker rats of different ages. Animals were treated with both 37.5mg/kg MNU at 50 days of age and 2 x 15 mg/kg AOM at 57 and 64 days of age. n = 16 rats/group in fed-state groups and n = 12 rats/group in animals fasted 4 hours prior to blood sampling.

* Statistically significant from lean group, P<0.05.
to exhibit normal-mild hyperglycemia (Bray, 1977), the significantly higher serum glucose levels observed at 13 and 25 weeks of age in this experiment was probably due, in part, to the hyperphagia exhibited by obese rats (Bray, 1977). To verify the glucose levels independent of food intake in both groups, 12 rats/group were fasted 4 hours prior to blood sampling. As indicated in Figure 3.2, there was no significant difference in serum glucose levels between the two groups both at 19 and 25 weeks of age.

3.1.2.2 Serum Insulin Levels

Serum insulin levels for 7, 13, 19, and 25 week old fed-state animals and fasted 19 and 25 week old rats are shown in Figure 3.3. Obese Zucker rats are known to exhibit hyperinsulinemia whereas lean rats display normal insulin levels. As Figure 3.3 shows, fed-state serum insulin levels were significantly higher in obese rats compared to the leans at all ages measured. To separate the possible confounding effect of hyperphagia in the obese animals, rats were fasted 4 hours before blood sampling at 19 and 25 weeks of age. Under fasting conditions, significantly higher insulin levels were observed in the obese rats compared to the leans (P<0.0001).

3.1.2.3 Serum Triglyceride Levels

Hyperlipidemia is a characteristic of the obese Zucker rat, observed as early as 4 weeks of age (Bray, 1977; Zucker and Zucker, 1962). To monitor blood lipid levels in the animals used in the tumorigenesis experiment, fed-state serum triglyceride levels were
Figure 3.3  Serum insulin levels (μIU/mL) of lean (white bars) and obese (black bars) Zucker rats of different ages. Animals were treated with both 37.5 mg/kg MNU at 50 days of age and 2 x 15 mg/kg AOM at 57 and 64 days of age. n = 16 rats/group in fed-state groups and n = 12 rats/group in animals fasted 4 hours prior to blood sampling. Serum insulin levels at all ages (fed-state and fasted) in the obese group are statistically significant from the lean rats, P<0.0001.
measured at 7 and 11 weeks of age. As shown in Figure 3.4, serum TG levels were significantly higher in the obese Zucker rats compared to the leans at both 7 weeks (238.9 ± 21.6 versus 164.4 ± 20.6 mg/dL, respectively, P<0.02) and 11 weeks of age (315.7 ± 46.8 versus 202.8 ± 14.7 mg/dL, respectively, P<0.03).

3.1.3 Tumor Incidence and Multiplicity

To assess the susceptibility of Zucker rats to developing mammary and/or colon tumors, the rats were given a dose of 37.5mg/kg MNU followed by 2 weekly doses of 15mg/kg AOM. The obese and lean Zucker rats were observed to be susceptible to developing both mammary and colon tumors. Figure 3.5 shows the cumulative mammary tumor incidence for the leans and obese rats. The rate of tumor development was significantly higher in the obese rats than the leans (P<0.0003). Similarly, the final tumor incidence was significantly higher in the obese rats compared to the leans (83.3% versus 33.3%, respectively, P<0.0002). As Figure 3.6 shows, the mammary tumor multiplicity (i.e. tumors per tumor bearing rat (TBR)) was also significantly higher in the obese rats than the leans (2.32 ± 0.29 versus 1.30 ± 0.15 tumors/TBR, respectively, P<0.005).

In the colon, the final colon tumor incidence in the obese rats was significantly higher in the obese compared to the leans (77.8% versus 39.3%, respectively, P<0.004), as shown in Figure 3.7. Colon tumor multiplicity in the obese group was also significantly higher than the leans, as shown in Figure 3.8 (3.52 ± 0.48 versus 1.46 ± 0.25 tumors/TBR, respectively, P<0.001).
Figure 3.4 Serum triglyceride levels (mg/dL) of lean (white bars) and obese (black bars) Zucker rats at 7 and 13 weeks of age. Animals were treated with 37.5 mg/kg MNU at 50 days of age and 2 x 15 mg/kg AOM at 57 and 64 days of age. n = 16 rats/group.

* Statistically significant from lean group, P<0.02 at 7 weeks of age and P<0.03 at 13 weeks of age.
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Figure 3.5 Cumulative mammary tumor incidence in Zucker rats following treatment with 37.5mg/kg MNU. ■ lean, ● obese Zucker rats. n = 30 rats/group. The rate of mammary tumor incidence of the obese Zucker rats is significantly different from the lean group, P<0.0003.
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Figure 3.6 Mammary tumor multiplicity (mean ± SEM) in Zucker rats following treatment with 37.5 mg/kg MNU. n = 30 rats/group. *Statistically significant from lean group, P<0.004.
Figure 3.7 Final colon tumor incidence in Zucker rats following treatment with $2 \times 15\text{mg/kg AOM}$. * Statistically significant from lean rats, $P<0.005$. 
Figure 3.8 Colon tumor multiplicity (mean ± SEM) in Zucker rats treated with 2 x 15 mg/kg AOM.
* Statistically significant from lean group, $P<0.001$. 
3.1.4 Estrus Cycle Length

The estrus cycle length of normal rats is known to be approximately 4-5 days (Baker, 1979). Care was taken in this experiment to prevent the induction of pseudopregnancy by using gentle manipulation for vaginal smear sampling which would otherwise stop estrus cycling in the rats. Cycling was observed in all obese rats from which vaginal smears were taken. However, one lean rat displayed an irregular estrus cycle (i.e. remained in met-estrus) and was not included in the analysis. In this experiment, the mean estrus cycle length of the lean rats (5.7 ± 0.3 days) was not significantly different from that of the obese rats (5.2 ± 0.2 days) at 23-24 weeks of age, as shown in Figure 3.9.

3.2 DNA Methylation Dosimetry Study

3.2.1 Biologically Effective MNU Doses in Zucker Rats

To determine the biologically effective MNU dose, or the amount of MNU delivered to the epithelial cells of the mammary gland to initiate carcinogenesis, DNA methylation was measured in the mammary glands of 50 day old rats treated with various MNU doses. Results of the experiment are summarized in Figure 3.10. The level of DNA methylation in the mammary epithelial cells of obese rats treated with 37.5 mg/kg MNU was significantly higher than lean rats treated with the same MNU dose (23.1 ± 2.9 versus 12.4 ± 2.1 mmol 7-meG/mol DNA, respectively, P<0.02). DNA methylation levels in obese animals treated with 25mg/kg MNU were not significantly different from obese rats.
Figure 3.9 Mean estrus cycle length (mean ± SEM) in adult Zucker rats as determined by vaginal smears. n = 9 lean rats and 10 obese rats.
Figure 3.10  DNA methylation levels (mmol 7-methylguanine/mol DNA) in mammary epithelial cells of lean and obese Zucker rats at 50 days of age treated with various MNU doses. n = 5 rats/group. Groups not sharing similar letters are significantly different, P<0.05.
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treated with 37.5mg/kg MNU. However, obese rats treated with 20mg/kg MNU did result in significantly lower DNA methylation levels compared to obese rats treated with 25mg/kg or 37.5mg/kg MNU (9.4 ± 1.6 versus 19.7 ± 1.9 or 23.1 ± 2.9 mmol 7-meG/mol DNA, respectively, P<0.003). Lean rats treated with 37.5mg/kg MNU resulted in significantly higher DNA methylation levels than lean rats treated with 25 mg/kg MNU (12.4 ± 2.1 versus 6.0 ± 0.8 mmol 7-meG/mol DNA, respectively, P<0.02). DNA methylation levels of lean rats treated with either 25 mg/kg or 37.5 mg/kg MNU did not differ significantly from obese rats treated with 20 mg/kg MNU. However, lean rats treated with 25 mg/kg MNU had significantly lower DNA methylation levels than obese rats treated with either a 25 or 37.5mg/kg MNU dose (6.0 ± 0.8 versus 19.7 ± 1.9 or 23.1 ± 2.9 mmol 7-meG/mol DNA, P<0.0002 and P<0.0005, respectively). Similarly, lean rats administered 37.5 mg/kg MNU had significantly lower DNA methylation levels than obese rats treated with 25 mg/kg MNU (12.4 ± 2.1 versus 19.7 ± 1.9 mmol 7-meG/mol DNA, P<0.03).

3.2.2 Biologically Effective AOM Doses in Zucker Rats

DNA methylation levels were assessed in epithelial cells of the colonic mucosa of 57 days old lean and obese Zucker rats treated with 15mg/kg AOM to determine the biologically effective carcinogen dose delivered to the animals. As shown in Figure 3.11, DNA methylation levels (i.e. mmol 7-meG/mol DNA) were not significantly different between the lean and obese rats.

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Figure 3.11 DNA methylation levels (mmol 7-methylguanine/mol DNA) in colonic epithelial cells of lean and obese Zucker rats at 57 days of age treated with 15 mg/kg AOM. n = 5 rats/group.
CHAPTER FOUR

Discussion and Conclusions
Chapter Four

4. Discussion and Conclusions

4.1 Discussion

Environmental and dietary risk factors are known to play an important role in the etiology of breast and colon cancer. Diets high in fat have been shown in epidemiological and animal studies to be a positive risk factor of both cancers (Rose, 1986; Jain et al., 1980; Reddy, 1992; Carroll and Khor, 1984). However, there is controversy as to whether the tumor promoting effects of high fat diets are attributable to fat intake per se or to increased calories derived from the high fat intake (Carroll, 1986). Thus, excess energy intake has emerged as a possible determinant of mammary and colon cancer risk, as evidenced by work done in animal models (Thompson et al., 1985; Clinton et al., 1992). Caloric-restriction (e.g. 30-40% of calories) has consistently been shown to inhibit both mammary and colon tumorigenesis in animals (Klurfeld et al., 1987, 1989; Kumar, 1990). Furthermore, clinical studies have shown that excessive body weight is directly associated with breast and colon cancer risk (Helmrich et al., 1983; Giovannucci and Willet, 1994).

In addition to these risk factors, the digestibility of dietary carbohydrates has been shown to be a determining factor in the promotion or inhibition of colon and mammary cancer in the rat model (Caderni et al., 1991, 1993; Stamp et al., 1993; Hoehn and Carroll, 1979). Evidence from epidemiological studies have shown that simple carbohydrates are positively correlated with colon and breast cancer risk, whereas complex carbohydrates are inversely related (Centonze et al., 1993; La Vecchia et al., 1993; Franceschi et al., 1996). Despite the evidence for the role of dietary fat, energy intake/obesity, and dietary...
carbohydrate in mammary and colon carcinogenesis, the mechanism(s) to explain these effects are still unknown. However, Giovannucci (1995), McKeowyn-Eyssen (1994), Kazer (1995), and Stoll (1996) have all recently proposed that insulin or insulin resistance may be the mediator of the tumorigenic effects by these dietary risk factors. In support, epidemiological, animal, and in vitro evidence have suggested a possible promoting effect of insulin or insulin resistance on mammary and colon cancer development (Heuson et al., 1970, 1972; Tran et al., 1996; Corpet et al., 1997; Bruning et al., 1992; McKeowyn-Eyssen et al., 1996). Therefore, hyperinsulinemia and insulin resistance are possible risk factors for both cancers.

A potential model to study the extent to which these dietary risk factors may interact to modulate breast and colon cancer is the obese (fa/fa) Zucker rat. The obese Zucker rat is a well-characterized model of NIDDM and obesity, exhibiting hyperinsulinemia, insulin resistance, and hyperphagia (Bray, 1977). These metabolic abnormalities that arise from their genetic background allow for an examination of how dietary factors may act, individually or in combination, to determine their susceptibility to breast and colon carcinogenesis.

The first objective of the work in this thesis was to determine the susceptibility of the Zucker rat strain to breast and colon cancer development using a dual target organ protocol described by Shivapurkar et al. (1996). The advantages of the dual organ protocol include the absence of an interaction effect between MNU and AOM, and similar tumor latency for both mammary and colon tumors using the described dosage schedule (Shivapurkar et al., 1996). The use of MNU is preferable to DMBA in the tumorigenesis
study involving Zucker rats. The use of a water-soluble carcinogen such as MNU avoids any influence of the large adipose depot on the deposition and/or metabolic activation of DMBA. Furthermore, the promoting effects of common dietary risk factors for breast and colon cancer can be examined simultaneously in the Zucker rat using the dual organ protocol.

Since the use of the obese Zucker rat to study mammary and colon tumorigenesis had not yet been reported, it was necessary to document any effects of carcinogen treatment on the growth and development of NIDDM in these rats. The most obvious phenotypic characteristic of fa fa Zucker rats is their obesity. This feature of the Zucker rat strain was confirmed by the growth curves from the tumorigenesis study (Figure 3.1). The body weights of the obese Zucker rats were significantly greater than their lean littermates at all ages during the experiment. The results of the tumorigenesis experiment also confirmed the normal-mild glycemia documented in fa fa rats (Bray, 1977). As shown in Figure 3.2, serum glucose levels were assessed at 7, 13, 19, and 25 weeks of age in non-fasted animals and after a 4-hour fast. At 7 and 19 weeks of age, serum glucose levels were not significantly different when measured in non-fasted animals. However, obese Zucker rats had significantly higher glucose levels than lean rats at 13 and 25 weeks age under non-fasting conditions. The lack of a difference in serum glucose levels in lean and obese animals fasted 4-hours prior to blood sampling at 19 and 25 weeks of age indicated that the mild hyperglycemia observed in non-fasted obese Zucker rats was likely due to hyperphagia. Increased food intake is a well-documented feature in obese rats (Bray and York, 1972). The serum glucose levels show that obese rats in our
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tumorigenesis study had near-normal glycemia, confirming the results of previous studies (Bray, 1977; Herberg and Coleman, 1977).

Another unique feature of obese Zucker rats is their hyperinsulinemia and insulin resistance. As shown in Figure 3.3, obese Zucker rats had a significantly higher serum insulin level than the lean rats at all ages in a non-fasted state. As with the serum glucose levels, hyperphagia probably contributed to the markedly high insulin levels in obese rats. This is suggested by a decrease in serum insulin levels in obese rats following a 4-hour fast at 19 and 25 weeks of age. However, these serum insulin levels in obese rats were still significantly higher than 19 and 25 week old lean rats fasted for 4 hours. The hyperinsulinemia in fa fa rats coupled with the normoglycemia observed suggests that the obese Zucker rats in our experiment were insulin resistant. Both hyperinsulinemia and insulin resistance were maintained throughout the period of the study in the obese rats compared to their lean counterparts. The results are, therefore, in good agreement with the metabolic abnormalities previously documented in the obese Zucker rat (Bray, 1977; Herberg and Coleman, 1977; Zucker and Antoniades, 1972).

Although the obese Zucker rat is commonly used as a model to study obesity and NIDDM, the use of these rats in tumorigenesis studies has been rare. The susceptibility of the background strains of Zucker rats (i.e. Merck Stock M and Sherman rats) towards mammary or colon carcinogenesis have not been determined. It was speculated that the Zucker rat strain may be resistant to either or both mammary and colon cancer since susceptibility to carcinogen-induced mammary cancer as well as colon cancer has been known to vary widely among different strains of rats (Issacs, 1986, 1988; Gould, 1986;
Takizawa et al., 1978; Asano et al., 1978). The initial tumorigenesis experiment, however, showed that both the lean and obese Zucker rats are susceptible to the development of mammary and colon tumors (Figure 3.5-3.8). As Figure 3.5 and 3.6 indicate, obese rats had a significantly higher rate of mammary tumor appearance, final tumor incidence, and mammary tumor multiplicity (i.e. number of tumors per TBR). Similar results were observed in the colon, where the final colon tumor incidence and multiplicity was approximately two-fold higher in the obese rats than in the lean Zucker rats (Figure 3.7 and 3.8). Therefore, the obese Zucker rat seems to be more susceptible to developing both mammary and colon cancer. The increased susceptibility of fa/ff Zucker rats towards mammary carcinogenesis is perhaps not surprising since the LA N-cp (corruptent) rat, a genetically obese rat with a similar leptin receptor mutation (Yen, 1977; Kahle, 1997), has recently been shown to develop DMBA-induced mammary tumors (Klurfeld et al., 1991). Furthermore, preliminary results from Weber et al. (1998) suggest that obese rats are also more susceptible to developing ACF than lean Zucker rats.

The second objective of this thesis was to determine the relative susceptibility of lean and obese Zucker rats to mammary gland and colon carcinogenesis. Although the results of the tumorigenesis experiment show that the obese Zucker rat is more susceptible than the lean Zucker rat to both mammary and colon cancer development, one possible confounding factor is the difference in body composition between lean and obese Zucker rats. At the time of carcinogen treatment, the body weights of the fa/ff were significantly greater than the lean rats, as shown in the growth curves of these animals (Figure 3.1). Since MNU and AOM are both water-soluble carcinogens, the biologically effective
carcinogen dose, or the amount of carcinogen delivered to the target tissue to initiate carcinogenesis, is highly dependent on body composition. Specifically, the lean tissue mass contains the target epithelial cells for MNU and AOM. However, a large proportion of the excess body weight in the obese Zucker rat is adipose tissue mass, as indicated by their increased efficiency of food utilization towards fat deposition as compared to lean Zucker rats (Bray, 1970; Bray et al., 1973; Deb and Martin, 1976; Zucker, 1975). Therefore, at any given total body mass, fa/fa rats consistently have a lower proportion of lean tissue weight than their lean littermates. Consequently, by simply administering water-soluble carcinogens based on total body weight, the obese Zucker rats may have received a higher biologically effective MNU and/or AOM dose than the lean rats in the initial tumorigenesis experiment. In such a case, any differences in breast and colon tumorigenesis between obese and lean animals would be enhanced. Hence, a difference in susceptibility towards carcinogenesis as a result of the metabolic abnormalities in fa/fa rats, such as obesity/excess caloric intake and insulin/insulin resistance, would be masked.

To calculate the biologically effective carcinogen dose, an approximation can be made using Refinetti’s theoretical model for the computation of the effective body mass or the metabolically active tissue mass for Zucker rats (Refinetti, 1989). The aim of Refinetti’s mathematical model is to allow for comparisons between lean and obese rats in metabolic studies, since the greater body mass of obese rats does not allow for a direct comparison. Furthermore, since body composition is different between lean and obese rats, the metabolic rate per unit of body mass is also not a reasonable method of comparison. Using Refinetti’s model (1989), the effective body mass for lean and obese
Zucker rats can be calculated as $1.00 \, M^{0.75}$ and $0.82 \, M^{0.75}$, respectively, where $M$ is the mass of the animal. According to these equations, the effective MNU and AOM dose administered to obese rats was estimated to be 30%-40% greater than that given to the lean Zucker rats. From these calculations, differences in body composition of obese and lean Zucker rats indeed have a significant effect on the biologically effective carcinogen dose.

In order to determine the biologically effective MNU and AOM dose delivered to the target tissues of the animals in the tumorigenesis experiment, DNA methylation levels resulting from carcinogen exposure were measured in the mammary gland and colonic epithelial cells of Zucker rats. Methylating agents such as MNU and AOM form DNA adducts which are thought to generate a somatic mutation that initiates the development of cancer. Thus, formation of DNA adducts serves as a biomarker of the initiating event by carcinogens, including MNU and AOM (Singer, 1975; Saffhill et al., 1985). Specifically, 7-meG levels in the DNA from mammary gland and colonic epithelial cells were quantitated in the DNA methylation dosimetry experiment. Although $O^6$-methylguanine ($O^6$-meG) is the DNA adduct associated with mutagenesis and carcinogenesis, 7-meG is the major DNA adduct formed from MNU and AOM exposure (Singer, 1975; Saffhill et al., 1985). The ratio of 7-meG:$O^6$-meG has been shown to be relatively consistent (approximately, 10:1) for a given carcinogen dose, making 7-meG a reasonable estimate of total DNA methylation by carcinogens and consequently, an indirect indicator of carcinogenic potency (Lawley, 1974; Pegg, 1977). Furthermore, the higher level of 7-meG gives better sensitivity in detection by HPLC than $O^6$-meG.
The results of the DNA methylation dosimetry experiment showed that there was a significantly higher level of 7-meG in the mammary glands of obese Zucker rats than lean rats treated with 37.5mg/kg MNU (Figure 3.10). This result indicated that the MNU dose used in the tumorigenesis experiment led to a significantly higher biologically effective carcinogen dose in obese Zucker rats. Based on the DNA methylation levels, obese rats received approximately a 40% higher MNU dose than the lean rats. The higher measured biologically effective MNU dose given to fa/fa rats is, therefore, in good agreement with that predicted from Refinetti's theoretical model (Refinetti, 1989) for the determination of the effective body mass. In order to determine the MNU dose which results in the same 7-meG level in both lean and obese Zucker rats, an experiment using various MNU doses was performed. From Figure 3.10, it is clear that an MNU dose of 20mg/kg administered to obese Zucker rats results in a comparable DNA methylation level as lean rats given a 37.5mg/kg MNU dose, suggesting similar biologically effective carcinogen doses. In light of this result, it would seem that obese Zucker rats may not be more susceptible than lean rats towards developing mammary cancer. Therefore, future tumorigenesis studies using the appropriate MNU doses must be used to re-evaluate the susceptibility of fa/fa Zucker rats to mammary gland carcinogenesis.

In contrast to the mammary gland, lean and obese Zucker rats treated with a 15mg/kg AOM dose showed similar 7-meG levels in the DNA of colonic epithelial cells (Figure 3.11). This suggests that this dose of AOM was an equal biologically effective dose for both groups of animals. The absence of a difference in DNA methylation levels in the colon was somewhat surprising given the observations in the mammary gland.
Although AOM and MNU administered i.p. are known to initiate carcinogenesis primarily in the colon and mammary gland, respectively, they are different in their mode of initiating carcinogenesis. MNU is a direct-acting carcinogen whereas AOM requires metabolic activation in the liver to form the ultimate carcinogen. Therefore, the degree of initiation in the colon by AOM is dependent on the extent of \textit{in vivo} transformation of AOM to a reactive, mutagenic species in the liver.

As discussed in Section 1.2.4.2 and shown in Figure 1.1, AOM is hydroxylated in the liver to MAM by the cytochrome P_{450}-dependent mixed function oxidase (MFO) of the endoplasmic reticulum (Hamilton et al., 1988). It has been shown that the liver P_{450} enzyme CYP2E1 is responsible for the metabolic activation of both AOM and its proximate metabolite MAM (Sohn et al., 1991; Sohn and Fiala, 1995). Increased rates of liver metabolism of AOM and MAM has been reported with the induction of CYP2E1 (Sohn et al., 1987; Fiala et al., 1987). Consequently, the availability of MAM for transport from the liver to the colon via the bloodstream to form the ultimate carcinogen is decreased when CYP2E1 is induced (Fiala et al., 1991). A greater amount of MAM metabolized in the liver results in a reduced amount of MAM available for transformation to the ultimate carcinogen in the colon (Fiala et al., 1991; Sohn and Fiala, 1995).

Sohn and Fiala (1995) have shown that a three-week 30% dietary restriction and a two day fast enhances CYP2E1 activity in the liver. This effect was reflected in increased AOM-induced liver DNA methylation as compared to controls and lower levels of DNA methylation in the colon (Sohn and Fiala, 1995). Thus, one possible explanation for the lack of a difference in colonic DNA methylation levels in lean and obese Zucker rats.
treated with 15mg/kg AOM may be an enhancement of P_{50} enzyme activity in livers of obese rats. A decreased action of insulin on peripheral insulin-sensitive tissues (i.e. insulin resistance), such as the liver and muscle tissue, results in metabolic changes similar to fasting conditions (Granner and O'Brien, 1992). The anabolic action of insulin to increase glucose uptake is depressed during a state of insulin resistance. Consequently, defective, unrestrained hepatic glucose production occurs as a result of insulin's inability to regulate gluconeogenesis properly. Furthermore, hyperinsulinemia occurs as a compensatory response to peripheral insulin resistance so as to maintain glucose homeostasis. Nevertheless, at a cellular level, glucose uptake is compromised, explaining the development of hyperglycemia and glucose intolerance in the obese Zucker rat. The insulin resistant state in NIDDM thus results in metabolic alterations that parallel fasting conditions, which may act to enhance the activity of P_{50} enzyme levels in the liver, including CYP2E1. Hence, in obese rats, a greater proportion of AOM and MAM would be metabolized in the liver. Consequently, lower MAM availability from the liver will limit the amount of DNA methylation that occurs in the colon of obese rats (Fiala et al., 1991; Hamilton et al., 1988; Hong and Yang, 1985).

Results from the DNA methylation dosimetry experiment with MNU showed that a dose of 37.5mg/kg was a significantly higher biologically effective carcinogen dose in obese than in lean Zucker rats. Based on this data, an AOM dose of 15mg/kg is also likely to be a greater biologically effective carcinogen dose in obese rats than in lean Zucker rats. However, the effect of AOM to initiate carcinogenesis in the colon of obese rats is likely normalized by the metabolic activation mechanisms in the liver, as discussed above. For
this reason, no significant differences were observed in the DNA methylation dosimetry experiment using 15mg/kg AOM in obese and lean Zucker rats.

Unlike the colon, mammary tumors in rats are strongly sex hormone-dependent for both induction and growth (Welsch, 1985). Due to the metabolic abnormalities of the fa:fa rat, any significant changes in sex hormone levels may have contributed to susceptibility of obese Zucker rats towards mammary carcinogenesis. This seems unlikely, however, since the length of the estrus cycle of adult obese rats was not significantly different from the leans (Figure 3.9). Although the length of the estrus cycle was examined only in adult Zucker rats, both lean and obese rats have been reported to have comparable levels of circulating estrogen (Bivens et al., 1997), LH and FSH (Saiduddin et al., 1973). This suggests that any changes in sex hormones are minimal and likely did not contribute a great extent to the increased susceptibility of obese Zucker rats towards mammary carcinogenesis observed in the tumorigenesis study.

The results of the tumorigenesis study and the DNA dosimetry study together suggest obese fa:fa Zucker rats are more susceptible to developing colon cancer than lean rats. However, it is unclear if this is also the case for breast cancer development in the obese Zucker rat. Increased susceptibility of the obese Zucker rat towards colon carcinogenesis may be the result of several factors. Firstly, there has been evidence to suggest that insulin promotes colon cancer development. Tran et al. (1996) have shown that exogenous insulin promotes colon tumor development in F344 rats initiated with AOM. In that study, insulin was given exogenously in a manner that simulated the high levels of insulin that occur after boluses of rapidly absorbed carbohydrates in animals.
(Tran et al., 1996). Using a similar protocol, Corpet et al. (1997) have reported that exogenous insulin promotes the growth of ACF in AOM-treated rats. However, since hyperinsulinemia is a compensatory response to the development of insulin resistance, it is also possible that insulin resistance leads to colon cancer promotion. In the obese Zucker rat, insulin resistance may, therefore, contribute to their increased susceptibility to colon carcinogenesis compared to lean rats. Koohestani et al. (1997) have shown that a high fat diet leads to the development of insulin resistance (as assessed by an OGTT) while at the same time promoting the development of ACF in AOM-initiated rats. Although insulin resistance was detected in this study by Koohestani et al., serum insulin levels were not consistently elevated, suggesting that insulin resistance, rather than hyperinsulinemia, is a marker for colon cancer risk (Koohestani et al., 1997). Although the mechanism by which insulin resistance may promote colon cancer development is not clear, it may nevertheless increase the susceptibility of obese fa·fa Zucker rats towards colon carcinogenesis.

The most prominent feature of the fa·fa rat is their obesity, which is the result of both an increase in energy intake and an elevation in the efficiency of food utilization. As discussed in Section 1.2.3.1 and 1.2.3.2, the obesity is due, in part, to a mutation in the leptin receptor involved in the regulation of food intake. Since obesity and caloric intake have been shown to be important factors in modulating colon cancer development (Clinton, 1992; Kumar et al., 1990; Steinbach et al., 1993), it is possible that excess caloric intake in fa·fa rats increase their susceptibility towards colon carcinogenesis. Preliminary results from Weber et al. (1998) have shown that obese Zucker rats, when initiated with AOM, develop a significantly greater number of colonic adenocarcinomas.
and aberrant crypts than lean control Zucker rats. These results confirm the findings presented in this thesis. Since \textit{fa:fa} rats fed a low fat diet was promoted more than lean Zucker rats fed a high fat diet of 20% corn oil + 20% lard or 40% lard, the authors concluded that obesity, more than dietary fat, promotes colon cancer in Zucker rats (Weber et al., 1998). Although preliminary, these results do suggest that obesity or excess caloric intake may increase the susceptibility of \textit{fa:fa} rats towards colon cancer development.

In contrast to the colon, the susceptibility of the obese Zucker rat towards mammary carcinogenesis is not clear from the results of the tumorigenesis and DNA methylation dosimetry study. Determination of the susceptibility of \textit{fa:fa} Zucker rats towards breast cancer is made difficult by their obesity, since the results of the DNA methylation dosimetry study suggest that body composition plays an important role during initiation by MNU. Despite this, work done by Klurfeld et al. (1991) in the genetically obese \textit{LA N-cp (corpulent)} rat has shown that DMBA-induced tumorigenesis is enhanced. Since the DMBA dose in this particular study was given in an absolute amount, independent of body weight, obese rats received a much lower dose of carcinogen per unit body mass than the lean animals. Mammary tumor incidence, however, was significantly greater in the obese rats even though a lower DMBA dose was administered (Klurfeld et al., 1991). In light of the discovery of a similar defect in the leptin receptor of both obese Zucker and \textit{LA N-cp corpulent} rats (Kahle et al., 1997), it is not unreasonable to expect enhanced mammary tumorigenesis in \textit{fa:fa} rats treated with a biologically effective MNU dose equal to that administered to lean Zucker rats.
Klurfeld et al. (1991) have further suggested that insulin in addition to adipose and lean tissue mass is a determinant of mammary tumor promotion in LA\(N\)-cp (corpulent) rats. Hence, a possible increased susceptibility of mammary carcinogenesis in obese Zucker rats may be due to elevated insulin levels. However, a recent study by Lu et al. (1998) showed that exogenous insulin does not promote tumorigenesis in MNU-initiated SD rats using a protocol previously shown to promote colon cancer development in AOM-treated rats (Tran et al., 1996). The discrepancy in response of the colon and mammary gland towards exogenous insulin (Tran et al., 1996; Corpet et al., 1997; Lu et al., 1998) may lie in the fact that administration of exogenous insulin does not simulate the prolonged insulin exposure and euglycemia associated with hyperinsulinemia and insulin resistance. Rather, this model simulates the high levels of insulin following boluses of rapidly absorbed carbohydrates in animals resistant to insulin (Tran et al., 1996). A consequence of using exogenous lente insulin is a large increase in serum insulin levels accompanied by a sharp decrease in blood glucose levels following the treatment. Changes in other hormone levels, such as growth hormone or glucagon, induced by the "spiking" insulin levels may account for the differential promoting effect in the colon and mammary gland. Thus, different mechanisms may exist for exogenous insulin's promoting effect in the two tissues. Since exogenous insulin does not precisely simulate the actions of endogenous insulin during an insulin resistant state, different effects on mammary carcinogenesis may also be observed with the two sources insulin. The obese Zucker rat can thus serve as a potential model to study the possible promoting effects of endogenous insulin on mammary and colon carcinogenesis.
Chapter Four

Excessive energy intake and obesity in \textit{fa\textasciitilde fa} rats may also be a possible factor in modulating the susceptibility towards mammary carcinogenesis, since studies have shown caloric intake to be a strong determinant of tumorigenesis. Thompson et al. (1985), for instance, have shown no difference in mammary tumor incidence in MNU-treated rats fed a high or low fat calorie-restricted diet, although \textit{ad libitum} feeding resulted in a higher tumor incidence in the high fat group. Caloric restriction has also been found to inhibit the growth of many types of tumors, including those of the breast (Klurfeld et al., 1987, 1989). In support of a possible role of obesity in the increased susceptibility of \textit{fa\textasciitilde fa} rats to breast cancer is a report that genetically obese and hyperinsulinemic yellow mice are more responsive to the tumorigenic effects of DMBA (Wolff et al., 1982). Future experiments (as discussed in Section 4.2), however, will determine which factors are indeed responsible for the possible enhanced susceptibility of obese Zucker rats towards the development of mammary gland carcinogenesis.

The obese Zucker rat is predisposed to obesity and the development of NIDDM as a consequence of its genetic background. Furthermore, the metabolic abnormalities observed in the \textit{fa\textasciitilde fa} rat resemble the syndrome of NIDDM in humans (Coleman, 1982), including hyperinsulinemia, insulin resistance, and obesity/excess caloric intake. The work presented in this thesis has shown that the Zucker rat strain is susceptible to breast and colon cancer development. Furthermore, the work presented has shown that the obese Zucker rat is more susceptible to colon tumorigenesis, although their susceptibility to breast cancer was not determined. Nevertheless, the obese Zucker rat may serve as a model to study the extent to which these metabolic abnormalities affect carcinogenesis so
as to increase their susceptibility to colon and possibly, breast cancer. These factors can act individually or in combination to enhance the susceptibility of obese Zucker rats to developing these cancers. Furthermore, the use of the dual organ protocol (Shivapurkar et al., 1996), using AOM and MNU, allows for the determination of the mechanism(s) responsible for the susceptibility of obese Zucker rats towards carcinogenesis simultaneously in two different tissues. As well, the identification of *Ki-ras* mutations and *Ha-ras* mutations in colon and mammary tumors induced by AOM and MNU, respectively, makes the obese Zucker rat a useful model to study diet-gene interactions. Chemopreventative agents, diet, and other lifestyle factors that may modulate colon and mammary carcinogenesis can be assessed using the obese Zucker rat. Therefore, although the obese Zucker rat has traditionally been used as a model of obesity/NIDDM, the work presented in this thesis suggests that the *fa/fa* rat may be a useful model to study colon cancer and possibly, breast cancer development.

### 4.2 Future Directions

The work in this thesis has shown that Zucker rats are susceptible towards developing breast and colon cancer. Furthermore, obese Zucker rats are more susceptible to colon carcinogenesis than lean rats. Differences in susceptibility towards breast cancer between lean and obese Zucker rats, however, can not be determined from the work presented. Therefore, future work should clearly include a tumorigenesis study with MNU-initiated obese and lean Zucker rats using equivalent biologically effective
carcinogen doses. The MNU doses used should be based on the data from the DNA methylation dosimetry experiment presented in this thesis, specifically a 20mg/kg dose in obese rats and a 37.5mg/kg dose in lean rats. In this experiment, the susceptibility towards mammary tumorigenesis in the obese Zucker rat can be observed independent of the effects of body composition on carcinogen dosage.

Although increased susceptibility of fa/ fa rats was only determined for colon cancer, it nevertheless suggests that various factors may be involved in their susceptibility. As discussed in Section 4.1, insulin and perhaps insulin resistance may increase the susceptibility of obese rats towards colon and perhaps mammary cancer development. To determine if hyperinsulinemia and insulin resistance does indeed promote carcinogenesis, it is necessary to account for the effects of hyperphagia or excess caloric intake on tumorigenesis in obese Zucker rats. Possible future experiments would include pair-feeding of obese Zucker rats to lean controls during a tumorigenesis study. Pair-feeding in long-term experiments has been shown to lower weight gain and insulin levels, although hyperinsulinemia and insulin resistance are maintained in the obese rats (Cleary et al., 1987). Therefore, this experiment will separate the effects of energy intake with that of insulin and insulin resistance.

To further study the role of insulin and insulin resistance on carcinogenesis, future work should include studies examining the effects of normalizing insulin levels on tumorigenesis in obese Zucker rats. Stolz and Martin (1982) have shown that STZ can be used to eliminate endogenous insulin production in obese Zucker rats. However, in this model, physiological doses of insulin must be administered to the animals to maintain
normal glucose levels (Stolz and Martin, 1982). The impractical aspects of this technique in a long-term tumorigenesis study makes the use of pharmaceutical agents to normalize insulin levels a more feasible option. For instance, diazoxide, which can be administered orally via the diet or intragastrically (i.g.), is a known insulin release blocking agent (Eaton and Schade, 1980; Gutman et al., 1985; Loubatieres et al., 1968; Porte, 1986). Furthermore, diazoxide has been shown to significantly inhibit tumor growth and volume of DMBA- and MNU-induced autochthonous rat mammary carcinomas (Berger et al., 1985). It would be interesting to further examine the effects of diazoxide on both mammary and colon tumor development in the obese Zucker rat.

Although the mechanism underlying the development of hyperinsulinemia and insulin resistance in the fa/fa rat is not entirely understood, there has been work to suggest that the insulin receptor is downregulated in both skeletal muscle and in the liver (Haring et al., 1984). Furthermore, other studies have reported a decrease in autophosphorylation of the IR tyrosine kinase (Slieker et al., 1990) as well as a lowered insulin-stimulated protein kinase C activity (Van De Werve, 1987). Thus, the evidence has supported both a receptor and post-receptor defect. Furthermore, this evidence suggests that insulin has an important role in modulating the insulin receptor. In light of this, it would be of interest to examine the IR status in both the mammary gland and colon. Upregulation of the IR in these tissues may provide a mechanism through which insulin can exert its promoting effects. Furthermore, key proteins, such as IRS-1 involved in insulin signalling pathways, and proteins known to affect the insulin signalling pathway, such as leptin (Cusin et al., 1995) will be of interest. Lastly, downstream pathways associated with insulin signalling
will also be important to consider. For instance, the ras pathway, known to be involved in insulin-mediated signalling (Jhun et al., 1994) has been implicated in mammary and colon carcinogenesis (Amundadttir and Leder, 1998; Gryfe et al., 1997).

4.3 Conclusions

The work in this thesis included two main objectives. The first aim of the work was to determine the susceptibility of the Zucker rat strain towards breast and colon cancer development. In the initial tumorigenesis study, it was observed that both lean and obese Zucker rats are susceptible to both mammary and colon tumorigenesis. It was hypothesized that the metabolic abnormalities that arise from the genetic background of obese Zucker rats, namely hyperinsulinemia, insulin resistance, and excess caloric intake, would predispose these rats to enhanced tumorigenesis. Therefore, the second objective of the work presented in this thesis was to determine if obese and lean Zucker rats are different in their susceptibility to mammary gland and colon cancer development when treated with carcinogens based on total body weight (i.e. mg/kg). Indeed, results from the tumorigenesis study suggested that fa fa rats are more susceptible to colon carcinogenesis than lean rats. This observation was confirmed by the DNA methylation dosimetry experiment showing that the biologically effective AOM doses administered to the animals in the tumorigenesis study were equivalent. However, the relative susceptibility of lean and obese rats towards mammary cancer development could not be determined by the tumorigenesis study. The DNA methylation dosimetry study revealed that different
biologically effective MNU doses were used in the tumorigenesis study, suggesting that the MNU dose is highly dependent on the body composition of the rats. Specifically, obese rats were shown to have received a higher biologically effective MNU dose than lean rats in the tumorigenesis experiment. Therefore, a re-evaluation of the relative susceptibility between obese and lean Zucker rats to mammary gland carcinogenesis, using equivalent biologically effective MNU doses, is warranted (as discussed in Section 4.2). Nevertheless, the increased susceptibility of the obese Zucker rat towards colon carcinogenesis has suggested that this rat may be a potential model to study the factors and/or mechanism(s) responsible for their enhanced susceptibility.
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