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DIET AND COLON PROTEIN KINASE C

Relation to intestinal tumour development in experimental animals

ACADEMIC DISSERTATION

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To my sons,
Tuomas and Juhana

ABSTRACT

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Colorectal cancer is one of the leading causes of cancer deaths in the Western countries and its incidence rates are currently increasing in all industrialised societies. Environmental factors including diet are considered to play a major role in colon tumourigenesis. A number of epidemiological studies suggest that diets high in fat and red meat and low in fibre rich foods, such as vegetables, fruits and cereals, are associated with an increased risk of colon cancer.

The aim of the present study was to elucide the mechanisms whereby diet may either prevent or promote colon tumour development. Specifically, it was examined whether fat, red meat, and different fibre sources are able to modify colonic PKC activity and isozyme expression in rat colonic mucosa. Furthermore, levels of PKC activators, such as fatty acids in mucosal phospholipids and diacylglycerol in colonic contents, were determined. The effects of fat, red meat, and fibre sources on intestinal tumour development were studied in APC^{Min} mice.

The results suggest that fats have no major effect on PKC activation or tumour development in the intestinal mucosa of rats and Min mice. However, feeding of a high-beef diet resulted in elevation of steady-state PKC activity in the colonic mucosa of rats as well as enhanced intestinal tumour development in Min mice. The effects of different fibre sources on PKC and tumour formation differed depending on the fibre type in question. The readily fermentable fibre sources inulin and oat bran resulted in an increase in PKC activity and PKC β2 expression in rat colon and enhanced tumour development in the intestine of Min mice, whereas wheat and rye brans resulted in low PKC activity and PKC β2 expression together with suppressed tumour development in Min mice.

CONTENTS

ABBREVIATIONS

AA	arachidonic acid
ACF	aberrant crypt foci
AIN	American Institute of Nutrition
AOM	azoxymethane
APC	human adenomatous polyposis coli gene
Apc	murine adenomatous polyposis coli gene
APC	adenomatous polyposis coli protein
COX	cyclooxygenase
DAG	diacylglycerol
DCC	deleted in colorectal carcinomas
DHA	docosahexaenoic acid (22:6 n-3)
EPA	eicosapentaenoic acid (20:5 n-3)
FAP	familial adenomatous polyposis
GSK3β	glycogen synthase kinase3β
HCA	heterocyclic amine
MAPK	mitogen-activated kinase
Min	multiple intestinal neoplasia
NF-κB	nuclear factor κB
NSAID	non-steroidal anti-inflammatory drug
PE	phosphatidylethanolamine
PC	phosphatidylcholine
PGE_2	prostaglandin E_2
PKC	protein kinase C
PLA_2	phospholipase A_2
PLD	phospholipase D
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
SCFA	short-chain fatty acid
SDS	sodium dodecylsulphate
Tcf	T cell factor

Abbreviations used in the original publications are not included and those used in the figures and tables are indicated in the legends.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles, which are referred to by their Roman numerals (I-VI) in the text:

I Pajari A-M, Rasilo M-L, Mutanen M. Protein kinase C activation in rat colonic mucosa after diets differing in their fatty acid composition. Cancer Lett 1997;114:101-103.

II Pajari A-M, Mutanen M. Phospholipid fatty acid composition and protein kinase C activity in the large intestine of rats fed on butter and coconut-oil diets. Br J Nutr 1999;82:411-418.

III Pajari A-M, Häkkänen P, Duan R-D, Mutanen M. Role of red meat and arachidonic acid in protein kinase C activation in rat colonic mucosa. Nutr Cancer 1998;32:86-94.

IV Pajari A-M, Oikarinen S, Duan R-D, Mutanen M. A high-beef diet alter protein kinase C isozyme expression in rat colonic mucosa. J Nutr Biochem 2000, in press.

V Pajari A-M, Oikarinen S, Gråsten S, Mutanen M. Diets enriched with cereal brans or inulin modulate protein kinase C activity and isozyme expression in rat colonic mucosa. Br J Nutr 2000;84:1-9.

VI Mutanen M, Pajari A-M, Oikarinen S. Beef induces and rye bran prevents formation of intestinal polyps in APC^{Min} mice: relation to β-catenin and PKC-isoforms. Carcinogenesis 2000;21:1167-1173.

In addition, some unpublished data are presented.

1. INTRODUCTION

Colorectal cancer is the fourth most common cancer in the world today (WHO 1997). Its incidence rates vary approximately 20-fold around the world so that the high-risk areas include North America, Europe, and Australia, whereas Central and South America, Asia, and Africa are areas of low risk. In Finland, cancer of the colon and the rectum are the second and third most common cancers in women and men respectively (Finnish Cancer Registry 2000). The number of new cases per year has steadily increased since 1965, being now three times higher than in the mid 1960s. The incidence rates of colon cancer are currently increasing in all industrialised countries as well as in the urban areas of developing countries (WHO 1997).

The 20-fold international difference in colon cancer rates is generally explained by differences in dietary habits and other environmental factors. In their much referred report, Doll and Peto (1981) estimated that dietary factors may account for approximately 35% of cancer deaths, although the range of their estimate was wide from as low as 10% to as high as 70%. Since the early 1980s, a large body of evidence has emerged from epidemiological studies concerning the relationship between diet and colon cancer, which was recently reviewed by the expert panel of the World Cancer Research Fund (1997). The expert panel concluded that the most effective way of preventing colorectal cancer is consumption of diets high in vegetables and low in red and processed meat. Furthermore, consumption of diets high in non-starch polysaccharides (fibre), starch and carotenoids and low in fat, sugar, and eggs possibly decreases the risk of colon cancer (World Cancer Research Fund 1997).

Colon tumourigenesis is a multistep process affected by both environmental and genetic factors. Over the past two decades, the genetic events involved in the initiation and progression of colon cancer have been identified and a molecular model for colon tumourigenesis has been proposed (Fearon and Vogelstein 1990, Kinzler and Vogelstein 1996). This model is currently widely accepted to explain the development of the majority of colon tumours. According to this model, normal epithelium progress to hyperplasia, early to intermediate to late adenoma, carcinoma, and finally metastasis (Figure 1). The driving force in the tumourigenic process is accumulation of mutations in key genes regulating cell growth, differentiation, and apoptosis, i.e. programmed

cell death. These key genes can be classified into two major classes: the proto-oncogenes and the tumour-suppressor genes. Activating mutations in proto-oncogenes generate oncogenes that typically induce cell proliferation and thus abnormal growth. The most frequently mutated oncogene in colon cancer is the *RAS* gene. Tumour-suppressor genes are needed for cell growth arrest and apoptosis and therefore mutational inactivation of these genes leads to loss of negative growth control. Among the tumour-suppressor genes important in colon cancer are the adenomatous polyposis coli gene (*APC*), the deleted in colorectal carcinomas gene (*DCC*), and the *p53* gene.

One of the major issues of current research is to understand how dietary factors interact with the genetic events to either enhance or suppress the tumourigenic process in the colon. Apart from carcinogens derived from environmental or cooking processes, diet as such is not likely to cause mutations in the key genes initiating colon carcinogenesis. However, the growth of both mutated and normal cells is regulated by different cell signalling pathways which may be either activated or suppressed by dietary constituents (Figure 1). One of these pathways consists of protein kinase C (PKC) isozymes which have been shown to be involved in colonic cell growth and differentiation as well as malignant transformation (Brasitus and Bissonnette 1998). Changes in

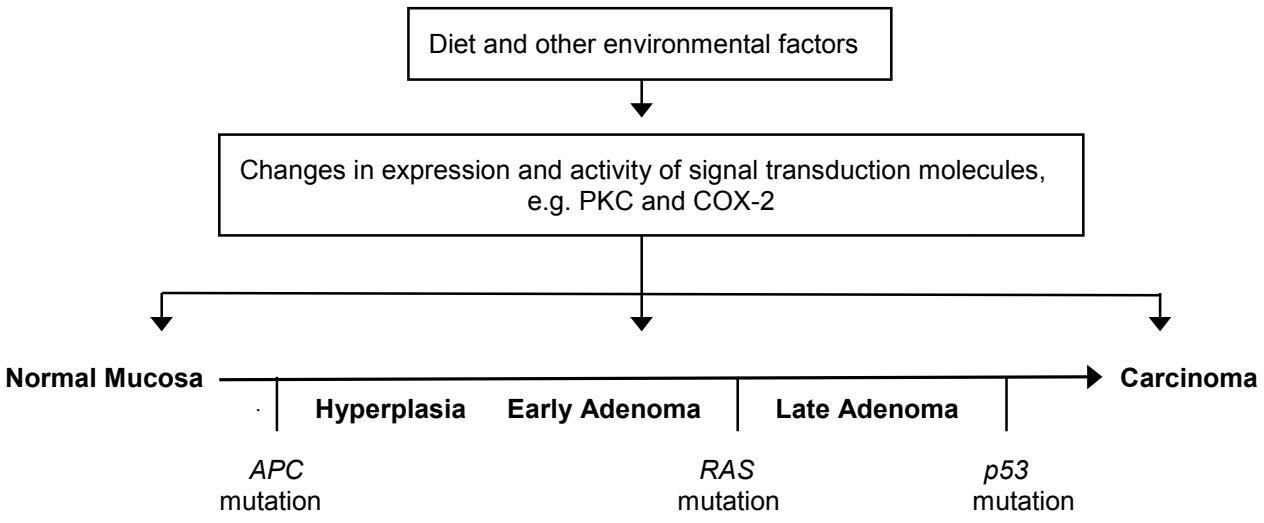

Figure 1. The adenoma-carcinoma sequence leading to colon cancer (modified from Fearon and Vogelstein 1990 and Williams *et al.* 1997).

PKC activity and isozyme expression are considered to occur at an early phase of colon carcinogenesis and therefore the dietary effects on PKC may be of importance. Cyclooxygenase-2 (COX-2) is generally recognised as perhaps even a more important contributor to colon carcinogenesis than PKC isozymes (Williams *et al.* 1997). However, changes in COX-2 expression and activity take place presumably somewhat later during the carcinogenic process when the possibilities of diet to modulate tumourigenesis are less promising. A number of other signal transduction pathways are likely to be involved in colon carcinogenesis. The sphingomyelin pathway is particularly interesting since it is capable of inducing apoptosis and dietary sphingomyelin may have a specific role in regulation of this pathway in the colon (Duan 1998). Furthermore, the APC/β-catenin pathway is of major importance because mutations in the *APC* gene are the initiating events for a vast majority of both inherited and sporadic colon cancer cases (Kinzler and Vogelstein 1996). Mutations in the *APC* gene lead to epigenetic changes in other members of the pathway, β-catenin in particular, and thus in proliferation, adhesion, and migration of intestinal epithelial cells (Pennisi 1998). There is also evidence that the APC/β-catenin pathway interacts with other cell signal pathways, for example with PKC isozymes (Murray *et al.* 1999).

This dissertation focuses on the mechanisms whereby diet may either promote or prevent colonic tumour formation. Special emphasis is laid on the dietary effects on the cell signal transduction pathways of PKC isozymes, sphingomyelinases, and APC-β-catenin.

2. CELL SIGNAL TRANSDUCTION PATHWAYS RELATED TO COLON CANCER

This section introduces those colonic signal transduction pathways that are within the scope of this dissertation. These pathways include the PKC, APC/β-catenin, sphingomyelin, and cyclooxygenase pathways. The PKC and APC/β-catenin pathways are described in detail because the major part of the experimental work in this dissertation concentrates on these two pathways. The sphingomyelin and especially cyclooxygenase pathways are introduced only briefly. The role of each pathway in controlling cell fate in the intestinal epithelium as well as their involvement in colon carcinogenesis is described. Figure 2 is an overview of these pathways and their possible interactions in colonic cells.

2.1 Protein kinase C

Description of the pathway

PKC comprises a large family of serine and threonine-specific kinases. At least 11 different PKC isozymes have been identified, of which colonic epithelial cells express α, β/β2, δ, ε, ζ, and ι/λ (Davidson *et al.* 1994, Kahl-Reiner *et al.* 1994, Wali *et al.* 1995). These PKC isozymes differ in their activator requirements so that PKC α and β/β2 are so called conventional isoforms activated by Ca^{2+}, diacylglycerol (DAG), and phosphatidylserine (PS). PKC δ and ε are novel isoforms activated by DAG and PS, whereas PKC ζ, and ι/λ are atypical isoforms stimulated by PS but not by Ca^{2+} or DAG. In a classical model for PKC activation, interaction of an extracellular signal with a receptor leads to formation of DAG and inositol triphosphate from cell membrane phosphatidylinositol. This, with a concomitant increase in intracellular Ca^{2+}, results in translocation of PKC from the cytosol to the membrane and subsequent activation of the enzyme (Nishizuka 1984). The events in the signal transduction proceed very rapidly and induce PKC activity only temporarily. Long-term modulation of PKC activity requires sustained production of PKC activators in cells, such as DAG via phospholipase D mediated breakdown of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) or free fatty acid and lyso-PC via phospholipase A mediated breakdown of PC (Nishizuka 1995). More recent studies have revealed that arachidonic acid (AA) and other *cis*-unsaturated fatty acids are also able to activate

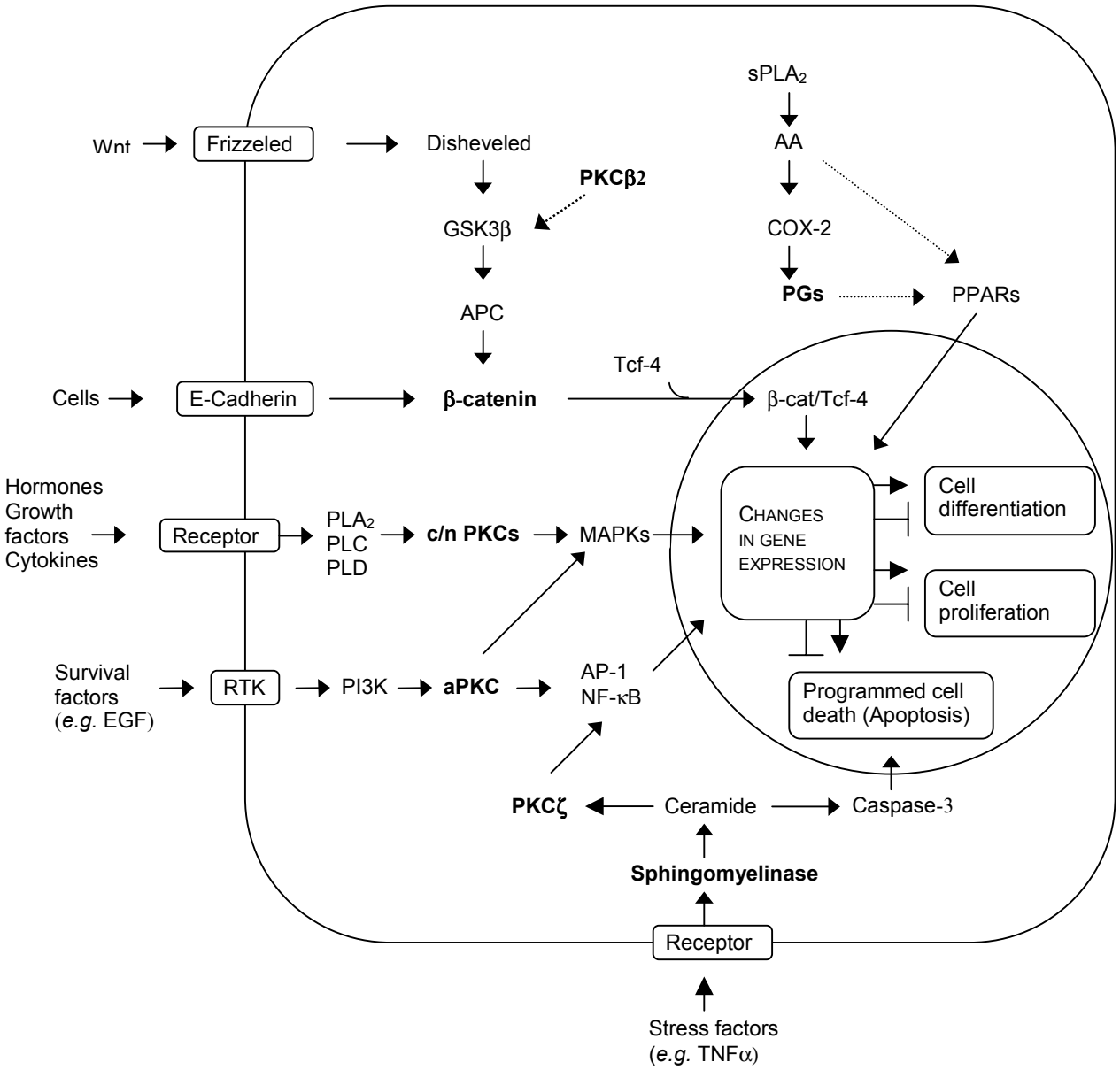

Figure 2. Cell signal transduction pathways related to colon carcinogenesis. The molecules analysed in the present study are indicated with bolding. AA, arachidonic acid; APC, adenomatous polyposis coli protein; aPKC, atypical protein kinase C; COX-2, cyclooxygenase-2; EGF, epidermal growth factor; GSK3β, glycogen synthase kinase 3β; MAPKs, mitogen-activated kinases; NF-κB, nuclear factor κ-B; PGs, prostaglandins; PI3K, phosphoinositide 3-kinase; c/nPKC, conventional/novel protein kinase C; $sPLA_2$,, secreted phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PPARs, peroxisome proliferator-activated receptors; RTK, receptor-tyrosine kinase, TNFα, tumour necrosis factor α.

PKC (McPhail *et al.* 1984, Murakami *et al.* 1986, Khan *et al.* 1991). Fatty acids preferentially activate soluble, cytosolic PKC and this activation is independent of or synergistic with the phospholipids and Ca^{2+}.

Variation in activator requirements, subcellular localisation, as well as tissue distribution enable PKC isozymes to regulate a wide range of cellular functions, such as cell proliferation and differentiation (Nishizuka 1995), cell cycle control (Thompson and Fields 1996, Livneh and Fishman 1997), intracellular trafficking (Sanchez *et al.* 1998) or even apoptosis (Emoto *et al.* 1996). The specific functions of PKC isozymes in colon epithelial cells are not yet fully understood. The evidence is most convincing in respect to PKC β2 and it suggests that PKC β2 mediates colonic cell proliferation. In colon carcinoma cells, overexpression of PKC β2 leads to blocked differentiation, restoration of the response for the basic fibroblast growth factor, and increased growth rate in athymic mice (Sauma *et al.* 1996, Sauma and Friedman 1996). These effects were accompanied by the constitutive activation of mitogen-activated kinases (MAPK).

PKC α appears to be involved in colonic differentiation and growth arrest (Frey *et al.* 1997, Abraham *et al.* 1998, Scaglione-Sewell *et al.* 1998). In human colon carcinoma CaCO-2 cells transfected with antisense PKC α, decreased PKC α expression was associated with increased proliferation, decreased differentiation, and a more transformed phenotype (Scaglione-Sewell *et al.* 1998). The possible mechanism whereby PKC α mediates its antiproliferative effect may involve induction of expression of $p21^{waf1}$, a cell cycle regulatory protein (Frey *et al.* 1997, Abraham *et al.* 1998). PKC α was shown to control the expression of adhesion molecules in human colon carcinoma cells, thereby supporting the role of PKC α in differentiation. (Chakrabarty *et al.* 1998). However, the study of Hochegger and co-workers (1999) argues against the growth inhibitory role of PKC α in the colon. Instead, oligonucleotides directed against PKC α and β resulted in inhibition of DNA synthesis, indicating that both isozymes induce proliferation.

The two novel isoforms PKC δ and ε seem to have opposite functions in colonocytes. A recent study with colon cancer cell lines demonstrated that activation of PKC ε triggers proliferative

signals, whereas activation of PKC δ leads to apoptotic signals (Weller *et al.* 1999). Immunohistochemical studies on PKC isozyme distribution along the colonic crypt axis also support the role of PKC δ in mediating postmitotic events in colonocytes *in situ* (Kahl-Rainer *et al.* 1996, Verstovsek *et al.* 1998).

Atypical PKCs ζ and λ can be activated by different growth factors (Akimoto *et al.* 1996, Liu *et al.* 1998) and cytokines (Müller *et al.* 1995, Limatola *et al.* 1997) and they have been involved in mediating growth, differentiation, and maturation in several cell types (Dominguez *et al.* 1992, Berra *et al.* 1993, Liu *et al.* 1998). Suggested downstream targets of PKC ζ include at least the κB nuclear factor (NF-κB; Lozano *et al.* 1994, Müller *et al.* 1995), the AP-1 transcription factor (Ways *et al.* 1994), and MAPKs (Berra *et al.* 1995). PKC ζ has also been shown to interact with the Ras protein in fibroblasts (Diaz-Meco *et al.* 1994) and to be involved in regulating the endocytic membrane transport of the epidermal growth factor receptor in HeLa cells (Sanchez *et al.* 1998). In normal rat colonic epithelium, PKC ζ has been found to be expressed predominantly in the postmitotic cells of the upper crypt and surface mucosa, supporting a role for PKC ζ in mediating maturation of colonocytes (Kahl-Rainer *et al.* 1996, Verstovek *et al.* 1998).

Role in colon cancer

A number of studies have shown that PKC activity and isozyme expression are changed during colon carcinogenesis. PKC activity in colon tumours of both human (Guillem *et al.* 1987, Kopp *et al.* 1991, Kusunoki *et al.* 1992, Levy *et al.* 1993) and animal origin (Baum *et al.* 1990, Wali *et al.* 1991, Craven and DeRubertis 1992) is reduced when compared with the surrounding uninvolved mucosa. In experimental animals, the levels of PKCs α, δ, and ζ have been down-regulated and the levels of β/β2 have been up-regulated in carcinogen-induced colon tumours (Craven and DeRubertis 1992, Roy *et al.* 1995, Wali *et al.* 1995, Jiang *et al.* 1997a). However, there are some inconsistencies in the human observations. Kahl-Rainer and co-workers (1994) reported reduced protein expression of all PKCs except β that was neither decreased nor increased. In contrast, the study by Davidson and co-workers (1994) showed increased levels of all the PKC isozymes in adenocarcinomas when compared with the normal mucosa. The increase was most pronounced in PKC β levels. One study demonstrated down-regulation of PKC β and ε

and up-regulation of PKC δ, while no change occurred in the levels of PKC α and ζ (Pongracz *et al.* 1995). This discrepancy in results between different studies is probably due to differences in sample taking as well as in experimental procedures to analyse PKC activity and expression.

Several studies indicate involvement of PKC β2 in malignant transformation of colonocytes. In carcinogen injected animals, both PKC β2 expression and membrane/particulate association were increased in tumours relative to the uninvolved surrounding mucosa (Wali *et al.* 1995), suggesting that membrane association of PKC β2 may be related to the growth advantage of tumour cells. This is supported by a recent *in vivo* study showing that transgenic mice overexpressing PKC β2 exhibit hyperproliferation in their colonic epithelium and have an increased susceptibility to azoxymethane(AOM)-induced aberrant crypt foci (ACF), preneoplastic lesions in the colon (Murray *et al.* 1999). PKC ε has also been implicated in the promotion of colon cancer. Perletti and co-workers (1996) showed that PKC ε could act as an oncoprotein when modestly overexpressed in nontumourigenic rat colonic epithelial cells. The same group also demonstrated with an *in vitro* model of colon carcinogenesis that PKC δ suppresses growth and reverses the transformed phenotype of the epithelial cells (Perletti *et al.* 1999). This result emphasises the potency of PKC δ in inhibiting the carcinogenic process. The exact role of atypical PKC ζ and λ in colonic tumourigenesis is less well known. However, the anti-inflammatory drug piroxicam was able to prevent the carcinogen-induced down-regulation of PKC ζ in rat colon, which was associated with reduction in tumour incidence (Roy *et al.* 1995).

In summary, there are abundant data to show the central role of PKC isozymes in the normal regulation of colonic proliferation and differentation as well as in malignant transformation. However, the specific cellular functions of all the isozymes in the colon are not yet fully understood, which makes the relation between PKC and colon carcinogenesis complex, though not less important.

2.2 Adenomatous polyposis coli/β-catenin

Description of the pathway

The following presentation of the APC/β-catenin pathway is mainly based on the reviews of Barth *et al.* (1997), Ilyas and Tomlinson (1997), Ben Ze`ev and Geiger (1998), Eastman and Grosschedl (1999), and Pennisi (1999). APC is a huge cytoplasmic protein that regulates cellular β-catenin levels. In the cytoplasm APC forms a multiprotein complex with two other proteins, glycogen synthase kinase 3β (GSK3β) and axin. In this complex, APC binds β-catenin, GSK3β phosphorylates both APC and β-catenin thereby strengthening their interaction as well as marking β-catenin for degradation. Axin binds simultaneously and directly with β-catenin, APC, and GSK3β, thus stimulating GSK3β-dependent phosphorylation and β-catenin degradation.

Wnt glycoproteins convey signals of growth and development between cells. The Wnt signalling antagonises the activity of the APC-GSK3β-axin complex, resulting in an increase in free cytoplasmic β-catenin. This signalling cascade is initiated by the binding of the secreted Wnt to the Frizzeled family of transmembrane receptors. These receptors activate the cytoplasmic phosphoprotein Dishevelled, which inhibits GSK3β activity, either directly or possibly through PKC β2 (Cook *et al.* 1996, Murray *et al.* 1999). Inhibition of GSK3β leads to accumulation of hypophosphorylated and thus stabilised β-catenin in the cytoplasm. Stabilised β-catenin relocalizes to the nucleus, where it binds to the transcription factor T cell factor-4 (Tcf-4), thereby enhancing expression of several genes involved in cell growth. The putative target genes of the β-catenin/Tcf-4 complex include the *c-MYC* oncogene (He *et al.* 1998), peroxisome proliferator-activated receptor δ (*PPAR*δ) gene (He *et al.* 1999), the *cyclin D1* gene (Tetsu and McCormick 1999), and others such as the connective tissue growth factor *WISP* genes (Pennica *et al.* 1998), the transcription factor c-*JUN* and *FRA-1* genes (Mann *et al.* 1999), and possibly the cell surface glycoprotein *CD44* gene (Wielenga *et al.* 1999).

In addition to the Wnt signalling, β-catenin also takes part in regulation of cell adhesion and motility. The cellular β-catenin pool mediating adhesion is located in adherens junctions at the plasma membrane. Adherens junctions are multiprotein complexes involved in cell-to-cell

contacts. The heart of this complex are transmembrane E-cadherin proteins. The cytoplasmic tail of E-cadherin binds β-catenin, which in turn complexes with α-catenin. α-catenin connects the E-cadherin-β-catenin complex to the actin cytoskeleton through vinculin. This linkage to the actin cytoskeleton is essential for the adhesion function of cadherins. Tyrosine phosphorylation of β-catenin has been implicated in the negative regulation of cell adhesion (Barth *et al.* 1997, Eastman and Grosschedl 1999). β-catenin can be tyrosine phosphorylated by the epidermal growth factor receptor and non-receptor tyrosine kinases, which may lead to disruption of interactions between β-catenin and cadherin or cadherin-catenin complex and the cytoskeleton (Hazan and Norton 1998, Rosato *et al.* 1998), and thus to decreased adhesion.

Role in colon cancer

The *APC* tumour suppressor gene is inactivated by somatic mutation in the majority of sporadic colorectal tumours (Kinzler and Vogelstein 1996). Germline mutations in the *APC* gene cause familial adenomatous polyposis (FAP), an autosomal dominantly inherited disease in humans that predisposes to colorectal cancer. FAP patients develop hundreds to thousands of adenomas predominantly in the lower gastrointestinal tract. If not removed, some of these tumours will progress to cancer. Based on these observations, *APC* is considered as the gatekeeper for colonic tumourigenesis. Altogether five murine models of human FAP have been created during the last ten years (Alexander 2000, Sasai *et al.* 2000). These mouse strains possess an induced germline mutation in the *Apc* gene which is a murine homologue of the human *APC* gene. Of these mouse models, the Min mouse (Min, multiple intestinal neoplasia) has been used most frequently to study the role of the Wnt/APC/β-catenin pathway in intestinal tumour development.

Inactivating mutation of the *APC* gene is an early, initiating event in colon carcinogenesis, resulting in expression of truncated APC protein. Mutant APC protein is unable to down-regulate β-catenin (Morin *et al.* 1997), which is critical for APC's tumour suppressor function. It is considered that elevation of cytosolic free β-catenin leads to enhanced translocation of β-catenin to the nucleus, its association with the Tcf-4 transcription factor, and thus abnormal transcriptional activity of the genes promoting cell proliferation. Indeed, colon carcinoma cells mutant for *APC* contain a stable β-catenin/Tcf-4 complex in the nuclei that is constitutively active (Korinek *et al.* 1997). Reintroduction of wild-type APC removed β-catenin from Tcf-4 and

inhibited transcriptional activation in these cells. In addition to *APC* mutations, activating mutations in the β-catenin gene can also result in elevated β-catenin/Tcf-4 mediated transcriptional activation (Morin *et al.* 1997). The role of β-catenin in colon tumourigenesis is further supported by studies showing that tumour cells from colorectal cancer patients exhibit reduced β-catenin in the cytoskeletal fraction and increased β-catenin in the cytosolic and nuclear fractions (Herter *et al.* 1999, Hugh *et al.* 1999).

APC and β-catenin may affect colonic tumourigenesis partly through their effects on cell adhesion and thus on cell-to-cell interactions, which is independent of the Wnt pathway regulating cell proliferation. In Min mice, the histologically normal intestinal epithelium revealed elevated β-catenin levels and changes in proliferation and apoptosis, which were associated with decreased rate of crypt-villus migration (Mahmoud *et al.* 1997). This abnormal migration could be due to disturbances in function of β-catenin/E-cadherin complexes in the adherens junctions. E-cadherin has been suggested to act as a tumour suppressor gene (Christofori and Semb 1999) and the loss of E-cadherin mediated cell adhesion has been demonstrated to be causally involved in the progression from adenoma to carcinoma *in vivo* (Perl *et al.* 1998). Because β-catenin is essential for the adhesion function of cadherins, changes in intracellular β-catenin distribution are likely to affect cell adhesion. The role of APC protein in adhesion is less clear though it is known that wild-type APC associates with the cytoskeleton by binding microtubules, while the mutated APC does not (Munemitsu *et al.* 1994). This suggests that APC may also be involved in β-catenin mediated cell-to-cell signalling.

2.3 Sphingomyelinase

Description of the pathway

Sphingolipids are located in cellular membranes and are important for the maintenance of membrane structure. Recent studies have revealed that sphingolipids, sphingomyelin in particular, may also serve as substrates for a cell signal transduction pathway related to cell growth suppression (reviewed by Duan 1998). Activation of sphingomyelinases results in hydrolysis of sphingomyelin and formation of ceramide, a putative lipid second messenger. Ceramide has been shown to induce apoptosis and cell cycle arrest (Obeid *et al.* 1993, Jayadev *et*

al. 1995) as well as inhibit cell proliferation and stimulate cell differentiation (Okazi *et al.* 1990, Hannun 1994). Ceramide may be further metabolised to sphingosine which can either be re-acylated back to ceramide or be phosphorylated to sphingosine-1-phosphate (Shimojo and Schroepfer 1976, Hassler and Bell 1993). Sphingosine inhibits growth and induces apoptosis (Hannun 1994, Jayadev *et al.* 1995), whereas sphingosine-1-phosphate promotes proliferation and inhibits apoptosis (Olivera and Spiegel 1993, Cuvillier *et al.* 1998).

Three types of sphingomyelinases differing in their pH optimum and cellular localisation have been identified. Acid sphingomyelinase is a lysosomal enzyme with an optimal activity at pH 5.0 (Spence 1993). Neutral sphingomyelinase presumably exists in two different forms. The classical form is a plasma membrane associated enzyme with a requirement for Mg^{2+} ions and a pH optimum at 7.4 (Chatterjee 1993). A soluble Mg^{2+} -independent neutral sphingomyelinase has been reported to be present in the cytosol (Okazi *et al.* 1994). Acid and neutral sphingomyelinases are expressed ubiquitously in mammalian tissues. Alkaline sphingomyelinase with a pH optimum of 9.0 is specific to the intestinal tract (Nilson 1969). Its activity is high in the small intestine and still considerable in the colon, with a decreasing gradient towards the rectum (Duan *et al.* 1996). Sphingomyelinases can be activated by a variety of extracellular agonists, such as tumour necrosis factor α, interferon-γ, interleukin-1β, and 1,25-dihydroxy vitamin D_3. Environmental stress factors including heat shock and ionising and ultraviolet radiation are also known inducers of sphingomyelin hydrolysis (for a review see Kolesnick and Krönke 1998). Ceramide produced by sphingomyelinases is thought to mediate its effects on cell functions through altering the phosphorylation status of proteins and thus the activity of transcriptional factors, for example inducing c-*MYC* down-regulation (Duan 1998). Ceramide produced by acid sphingomyelinase may have a specific role in activating atypical PKC ζ , which in turn leads to activation of the transcriptional factor NF-κB (Lozano *et al.* 1994, Müller *et al.* 1995). Ceramide might also inactivate PKC α by causing dephosphorylation of the enzyme (Jones and Murray 1995). Another member of the sphingomyelin pathway, sphingosine, is known to interact with PKC since an early study showed that sphingosine is an inhibitor of PKC activity (Hannun *et al.* 1986).

Role in colon cancer

Several types of findings indicate that sphingomyelinases may play an active role in colonic malignant transformation. Sphingomyelin levels are increased in human colon carcinomas as well as in the colonic mucosa of carcinogen-treated rats (Dudeja *et al.* 1986, Merchant *et al.* 1995). In FAP patients, the activities of all the three types of sphingomyelinases are reduced in colorectal carcinomas compared with the surrounding tissue (Hertervig *et al.* 1997). In mice treated with dimethylhydrazine, supplementation of the diet with sphingomyelin reduces the number of ACF and the appearance of adenocarcinoma in the colon (Dillehay *et al.* 1994, Schmeltz *et al.* 1997). Certain dietary constituents, such as dairy products and meat, contain sphingomyelin as well as other cell membrane phospholipids (Blank *et al.* 1992), which might affect colonic sphingolipid signalling and thus colon tumourigenesis. Although promising, the evidence regarding the role of sphingolipids in colon carcinogenesis is based on relatively few studies and therefore comprehensive conclusions are not yet possible.

2.4. Cyclooxygenase

Cyclooxygenase (COX) catalyses the conversion of arachidonic acid (AA) to prostaglandin endoperoxide H_2, which is the direct precursor of various prostaglandins, hydroxyeicosatetraenoic acids, and other eicosanoids. Two isoforms of COX exist: COX-1 is constitutively expressed in many tissues and cell types, whereas COX-2 is an inducible form frequently up regulated by mitogens, cytokines, and tumour promoters (Williams *et al.* 1997). The two isoforms have both overlapping as well as distinct physiological and pathological functions.

Role in colon cancer

Elevated COX-2 expression is currently presumed to play an important role in colon tumourigenesis. Human colon adenocarcinomas have been demonstrated to have increased COX-2 expression when compared to normal adjacent mucosa (Eberhart *et al.* 1994). These findings are supported by animal studies that have shown elevated levels of COX-2 in colon tumours of carcinogen-treated rats (DuBois *et al.* 1996) and intestinal adenomas of Min mice (Williams *et al.* 1996). Furthermore, treatment with sulindac, a non-steroidal anti-inflammatory drug (NSAID)

inhibiting both COX-1 and COX-2, markedly reduced tumour formation as well as decreased COX-2 expression and prostaglandin E_2 (PGE_2) levels in the intestine of Min mice (Boolbol *et al.* 1996). Celecoxib, a specific COX-2 inhibitor, suppressed AOM-induced tumour number and incidence in rats by more than 90% (Kawamori *et al.* 1998). These results suggest a causative role for COX-2 in colonic tumour promotion, which is probably mediated by enhanced production of PGE_2. The mechanisms of the tumour promotive action of PGE_2 may involve inhibition of apoptosis due to increased Bcl-2 protein levels (Sheng *et al.* 1998) and induction of angiogenesis (Tsujii *et al.* 1998). However, it is noteworthy that the tumour suppressive effect of NSAIDs may not be entirely mediated through COX inhibition because NSAIDs induced apoptosis in colon cancer cell line has been shown to be independent of PGE_2 (Earnest *et al.* 1997).

A number of studies have also suggested a close interaction between COX-2 and the APC-β-catenin pathway (reviewed in Prescott and White 1996). The most convincing evidence for this interaction comes from a study by Oshima and co-workers (1996). They introduced a knockout mutation of the *COX-2* gene to the $Apc^{\Delta 716}$ mice and found that the number and size of intestinal polyps in the mice with a disrupted *COX-2* gene were dramatically reduced when compared to mice with a fully functioning *COX-2* gene. It has been hypothesised that loss of both *APC* alleles at the early stage of the cancer process will induce COX-2 expression, leading to self-promotion of tumourigenesis (Prescott and White 1996).

3. EPIDEMIOLOGICAL AND EXPERIMENTAL EVIDENCE ON DIET AND COLON CANCER

This section gives a brief overview of the epidemiological and experimental evidence concerning the role of fat, red meat, and fibre rich foods in colon cancer. Because of the large body of evidence, only the studies considered to be most significant are referred here. The other dietary factors possibly associated with colon cancer are calcium, folates, vitamin D, vitamin E, carotenoids, and selenium. They are not within the scope of this work and are not reviewed here. Interested readers are referred to the World Cancer Research Fund report (1997). The purpose of this section is to offer a background to help the reader understand the mechanistic approach used in Studies I - VI of this dissertation.

3.1 Fat

Ecological studies, most of which were carried out in the 1970s, have reported a strong correlation between national per capita consumption of fat and mortality or incidence of colorectal cancer (see Potter *et al.* 1993). Based on these studies, the leading hypothesis until the beginning of the 1990s has been that dietary fat is the most important dietary risk factor for colon cancer. Most of the results from the earlier case-control studies have supported this hypothesis (Potter *et al.* 1993). However, a number of these studies did not distinguish fat intake from total energy intake. Fat and energy intakes are highly correlated with each other, and animal studies have shown that energy intake is a strong determinant of tumour growth in chemically induced colon carcinogenesis (Klurfeld *et al.* 1987). Therefore, the results of the earlier case-control studies may have been misinterpreted. A recent combined analysis of 13 case-control studies found no independent effect of fat on colon cancer risk after adjustment for total energy intake (Howe *et al.* 1997). Moreover, of the four prospective cohort studies that controlled for the total energy intake (Willet *et al.* 1990, Bostick *et al.* 1994, Giovannucci *et al.* 1994, Goldbohm *et al.* 1994), none found a significant positive association between total fat and the risk of colon cancer. In respect of the subgroups of fat, the Nurses' Health Study (Willet *et al.* 1990) reported a twofold higher risk of colon cancer in women in the highest quintile compared with those in the lowest quintile of animal fat intake. The other three studies did not find any association between

animal, vegetable, or polyunsaturated fat and the risk of colon cancer (Bostick *et al.* 1994, Giovannucci *et al.* 1994, Goldbohm *et al.* 1994).

A considerable number of experimental studies with rodent colon carcinogenesis model have been conducted to elucidate the effects of fat quantity and type on colonic tumour development. In some studies, diets high (20% by weight) in corn oil, safflower oil, beef tallow or lard have increased the incidence of colon cancer in carcinogen-treated rats compared with low fat diets (5% by weight) (Reddy 1992). No promoting effect on colonic tumourigenesis could be seen when rats were fed large amounts of olive oil, coconut oil, fish oil or hydrogenated fat high in trans fatty acids (Reddy 1992). For corn oil and beef tallow, there are experiments that have shown no increase in tumour incidence even though the level of these fats in diets has been 24% by weight (Nauss *et al.* 1983). The discrepancy in results between different studies is probably due to differences in the experimental procedures, including the species, strain, and sex of animals used. The type of carcinogen and its administration way have also varied among the studies. A quantitative analysis of 14 studies of rat colon carcinogenesis demonstrated a rather strong positive relationship between total fat intake and tumour incidence in F344 rats but not in Sprague-Dawley rats (Zhao *et al.* 1991). When the type of fat was taken into account, the results for both strains suggested a negative relationship between tumour incidence and n-3 fatty acids. A positive relationship between n-6 polyunsaturated fatty acids and tumour incidence was seen in F344 rats only, while monounsaturated and saturated fats were not associated with tumourigenesis (Zhao *et al.* 1991).

Dietary fats have also been studied in *Apc* mutated mice characterised by the development of spontaneous intestinal tumours along the entire intestine. In Min mice, a diet high in corn oil (15% by weight) and thus high in polyunsaturated n-6 fatty acids was found to increase adenoma number in both the small and large intestine (Wasan *et al.* 1997), supporting the results of some earlier rat studies. Two studies have examined the role of n-3 fish oil fatty acids in colon tumour development. In the first study, supplementation of AIN76A diet with 3% of docosahexaenoic acid (DHA) decreased tumour number in the intestine of $Apc^{\Delta 716}$ mice, though only in females (Oshima *et al.* 1995). In the second study, Min mice were fed varying amounts of eicosapentaenoic (EPA) and DHA enriched fish oil concentrate that was mixed with corn oil so

that the total oil content in the AIN76A-based diets was 12% (Paulsen *et al.* 1997). The fish oil concentrate reduced tumour number in both male and female mice when compared with the control group which had 12% pure corn oil in the diet (Paulsen *et al.* 1997). Taken together, recent epidemiological studies do not support a promotive effect of fat on colon cancer. Experimental studies strongly suggest that fish oil rich in n-3 fatty acids protects against colon cancer, whereas the role of saturated and n-6 fatty acids has been partly controversial.

3.2 Red meat

Although early ecological and case-control studies suggested that red meat consumption is positively associated with colon cancer risk, the promotive effect of red meat on colon carcinogenesis was mostly attributed to the fat content of meat. In the beginning of the 1990s, two large cohort studies in the U.S. demonstrated that intake of red meat is a stronger risk factor for colon cancer than total fat intake (Willett *et al.* 1990, Giovannucci *et al.* 1994). The Health Professionals Follow-Up Study, in particular, found a direct association between red meat consumption and colon cancer risk while no association was observed with total fat or animal fat (Giovannucci *et al.* 1994). These results have only partly been supported by the other recent cohort studies. In the cohort study from the Netherlands, consumption of processed meats but not fresh meat was related to an elevated colon cancer risk (Goldbohm *et al.* 1994), whereas four other cohorts showed no increase in risk with meat consumption (Thun *et al.* 1992, Bostic *et al.* 1994, Knekt *et al.* 1994b, Kato *et al.* 1997). The most recent cohort study reported an elevated risk in Seventh-day Adventists in association with higher intakes of both red and white meat (Singh and Fraser 1998).

So far seven studies have determined the effects of red meat on colon carcinogenesis in experimental animals. The results have been mixed and extremely difficult to interpret because of the differences in other components than the protein source of the experimental diets. An early study demonstrated that beef and soybean proteins did not differ in their effects on colonic tumour development. Feeding diets high in both beef or soybean resulted in an increased number of total adenomas and adenocarcinomas in rat colon compared with feeding their low-protein counterparts (Reddy *et al.* 1976). The studies by Nutter and co-workers (1983 and 1990)

suggested that milk proteins might be more tumour promoting than beef, but the experimental diets used in these studies differed in respect to type and amount of fat and therefore the comparisons between beef and milk proteins may not be valid. Three studies have controlled for the fat content of the diets. Of these studies, one examined the effects of red meat (kangaroo skeletal muscle), soybean meal, casein, and whey concentrate on colonic tumour development in rats and found that red meat- and soybean-fed animals had significantly higher number of intestinal tumours than those fed dairy proteins (McIntosh *et al.* 1995). The other two studies compared beef to casein, with results showing either no difference in tumour development between beef- and casein-fed rats (Lai *et al.* 1997) or a slightly suppressive effect of beef in a low-fat context (Pence *et al.* 1995a). A recent study by Parnaud and co-workers (1998) found no difference in multiplicity of colonic ACF in carcinogen-treated rats fed diets high in beef, chicken, or bacon. The limitation of this study is that though ACF are considered to be the earliest precursors of colonic tumours, their multiplicity has not always correlated with adenoma incidence (Hardman *et al.* 1991). Overall, the epidemiological findings are generally supportive of the promotive effect of red meat on colon carcinogenesis but experimental evidence for this is very inconsistent and limited.

3.3 Fibre rich foods

The hypothesis that high-fibre diets protect against colorectal cancer is several decades old and has been the subject of numerous studies. Case-control studies have generally supported the protective role of fibre in colon cancer. A combined analysis of 13 case-control studies demonstrated a strong, linear reduction in colon cancer risk with increasing fibre intake (Howe *et al.* 1992). Prospective studies have not confirmed the findings from case-control studies. Of four prospective studies, two found no association (Willett *et al.* 1990, Giovannucci *et al.* 1994) and two only a weak protective association (Heilbrun et al 1989, Steinmetz *et al.* 1994). Importantly, a very recent study containing a large cohort of nurses found no significant protective effect against colorectal cancer of total dietary fibre, or fibre from cereals, vegetables, or fruits (Fuchs *et al.* 1999). In this population, fibre from vegetables contributed most and cereal fibre least to the total fibre intake. One reason for the discrepancy between the case-control and prospective studies may be differences in study populations; all the prospective studies have been done in the

USA while populations in case-control studies have represented several nationalities with differing dietary practices. The other reasons for inconsistent results most likely involve the heterogeneous nature of fibre and fibre sources as well as differences in measuring fibre intake. It is also possible that other substances associated with fibre-rich foods, such as vitamins, minerals, phytate, and phytoestrogens, may account for the protective effect of fibre-rich foods. Whole-grain products are a rich source of both the bioactive compounds and fibre, and whole-grain intake has been associated with a reduced risk for colon cancer in two recent meta-analyses (Jacobs *et al.* 1995 and 1998).

The effects of different fibre sources on experimental colonic tumour development have been studied by using both carcinogen-treated rats and *Apc* mutated mice. The most studied fibre sources include cereal brans, cellulose, and fibres extracted from various vegetables and fruits such as carrots, sugar beets, and citrus fruits. Fruit and vegetable fibres are classified as soluble fibres, based on their water solubility, and grain fibres as insoluble fibres. Oat fibre is an exception since it contains considerable amounts of soluble fibre. The most consistent finding in experimental colon cancer studies has been that wheat bran prevents tumour formation in the colon of both rats (McIntry *et al.* 1993, Alabaster *et al.* 1995, Zoran *et al.* 1997) and *Apc* mutated mice (Hioki *et al.* 1997). The results for other fibre types have been less consistent but in general soluble fibres, such as oat bran, pectin, and guar gum, have increased rather than decreased the number and incidence of colonic tumours in animal models (Jacobs and Lupton 1986, McIntry *et al.* 1993, Zoran *et al.* 1997). Although whole grain rye products are consumed in large quantities in Northern and Eastern Europe, only one study of the effects of rye bran on experimental colon tumourigenesis has been published. In that study rye supplementation was shown to decrease colonic tumour development in carcinogen-treated rats (Davies *et al.* 1999). This result is expected since the composition of rye bran is close to that of wheat bran so that the two brans are rich sources of both fibre and bioactive compounds, e.g. phytate and phytoestrogens (Mazur and Adlercreuz 1998). Taken together, it seems that the protective effect of fibre is dependent on the fibre type and that high-fibre foods contain other constituents that may independently or in interaction with fibre contribute to colon cancer protection. This complex nature of the relationship between fibre rich foods and colon carcinogenesis may also explain the inconsistent results obtained in fibre studies.

4. PROPOSED MECHANISMS OF FAT, RED MEAT, AND FIBRE RICH FOODS IN COLON CARCINOGENESIS

4.1 Mechanisms related to fat

Over the past decades, modulation of intestinal bile acid metabolism and prostaglandin production have been the two leading hypotheses whereby dietary factors are thought to affect colon carcinogenesis (Figure 3). Especially fat is considered to act through these mechanisms but also the effects of red meat and fibre may be partly explained by bile acids and prostaglandins. Therefore this chapter contains some discussion concerning red meat and fibre although the major subject is fat.

4.1.1 Bile acids and protein kinase C

Bile acid metabolism

Over 95% of primary bile acids, cholic acid and chenodeoxycholic acid, are reabsorbed in the terminal ileum and returned to the liver through the portal vein (Nagengast *et al.* 1995). The remaining 2-5% continue into the colon where they are converted to secondary bile acids, deoxycholic acid and lithocholic acid, by anaerobic bacteria. The bacterial enzyme thought to be responsible for the conversion is 7α-dehydroxylase (Reddy *et al.* 1996). The secondary bile acids are postulated to have detrimental effects on the colonic epithelium and thus promote colon cancer. Animal studies have demonstrated that bile acids induce hyperproliferation of colonic epithelial cells (Lapre' and Van der Meer 1992) and promote colonic tumourigenesis (Reddy *et al.* 1977, McSherry *et al.* 1989). Studies with cancer cell lines indicate that the proliferative and thus the tumour promoting effect of bile acids is likely to be mediated by their ability to induce the activity of the transcription factor AP-1 (Hirano *et al.* 1996, Matheson *et al.* 1996, Glinghammar *et al.* 1999). An early human study by Reddy and Wynder (1973) also described a positive association between the incidence of colon cancer and faecal bile acid excretion.

Dietary fat is thought to promote tumourigenesis by increasing bile acid excretion, and thereby luminal concentrations of secondary bile acids in the colon (Figure 3, Potter 1993, Nagengast *et*

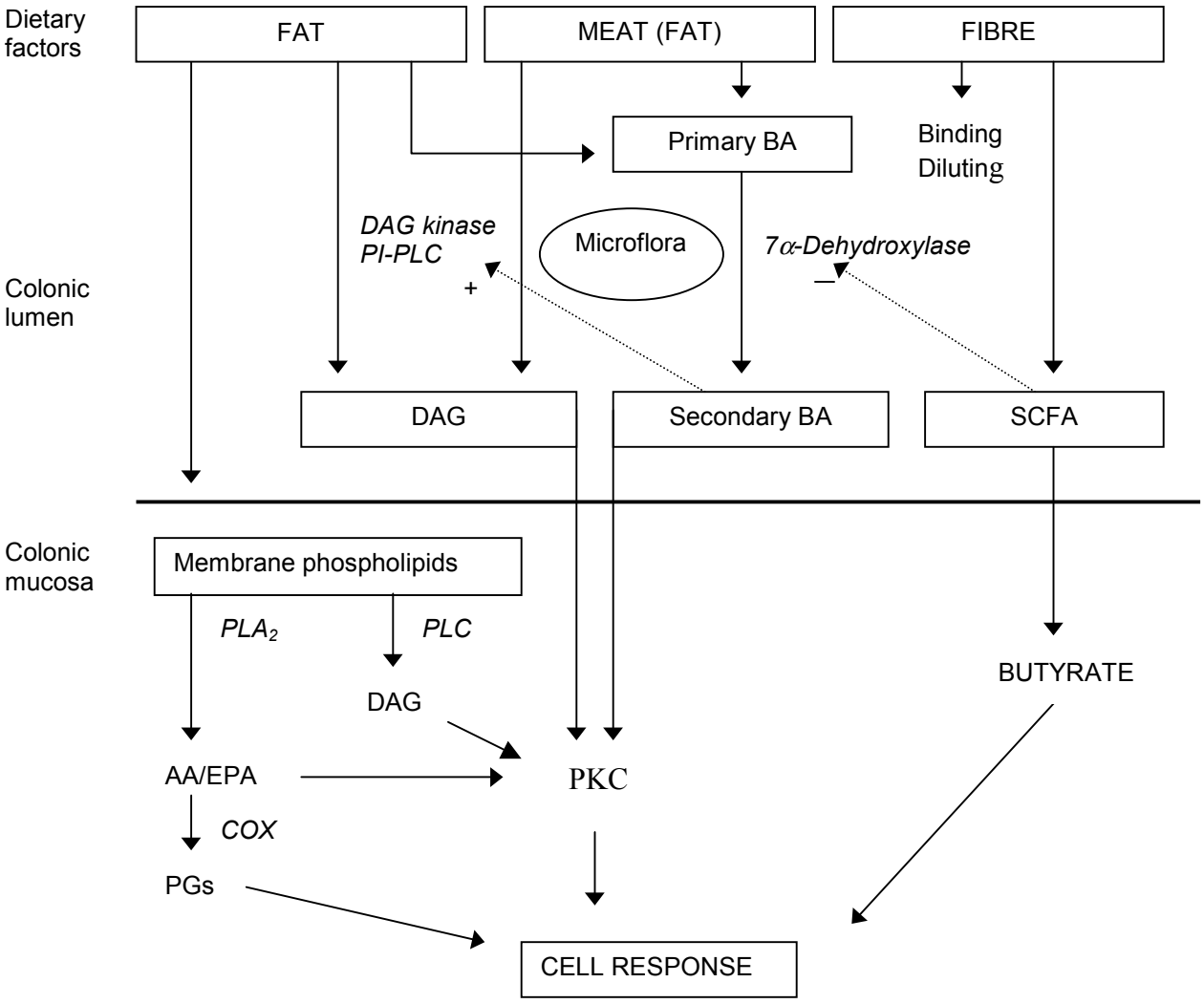

Figure 3. Possible mechanisms whereby dietary fat, meat, and fibre may affect colon tumour development (modified from Reddy et al. 1996). AA, arachidonic acid; BA, bile acid; COX, cyclooxygenase; DAG, diacylglycerol; EPA, eicosapentaenoic acid; PGs, prostaglandins; PLA_2, phopholipase A_2; PLC, phospholipase C; PKC, protein kinase C; SCFA, short-chain fatty acid.

al. 1995). Because diets high in meat tend to be high in fat, the relationship between red meat consumption and colon cancer has often been explained by the bile acid hypothesis. Dietary fibre may prevent colon tumourigenesis by increasing stool bulk, which binds and dilutes bile acids, and reduces transit time. This results in a shorter exposure of colonic epithelium to bile acids as

well as to other potentially toxic compounds. Bacterial fermentation of fibre produces short-chain fatty acids that decrease luminal pH and thereby inhibit the conversion of primary to secondary bile acids (Potter 1993). The experimental evidence concerning the bile acid hypothesis has not been entirely supportive. Though diets high in corn oil have usually resulted in elevated levels of secondary bile acids in faeces of experimental animals (Reddy *et al.* 1996), diets high in saturated fats, such as butter and beef tallow, have not increased faecal bile acids (Chang *et al.* 1994, Morotomi *et al.* 1997).

Bile acids and PKC

A more recent suggestion is that the interaction of fat, bile acids, and bacteria results in an excessive production of luminal DAG, a physiological activator of PKC (Morotomi *et al.* 1990). Indeed, it has been demonstrated that the colonic bacteria of human faecal specimens produce DAG and this production is enhanced by the presence of bile acids, the secondary bile acid deoxycholic acid being most potent in this respect (Morotomi *et al.* 1990). Luminal DAG was also taken up by mucosal cells (Morotomi *et al.* 1991), indicating that luminal DAG would be able to activate mucosal PKC pathway and thus stimulate cell proliferation.

Bile acids may also stimulate mucosal PKC activity directly. This is supported by the observations that both primary and secondary bile acids are potent activators of PKC extracted from human colon tissue samples (Pongracz *et al.* 1995) and that cholic acid feeding activates mucosal PKC in rats (Pence *et al.* 1995b). Bile acids are also known to activate PKC in colon cancer cell lines (Huang *et al. 1992,* Hirano *et al.* 1996). There is some evidence that bile acids may be able to stimulate the activities of mucosal PLC (Nomoto *et al.* 1994) and PLA_2 (DeRubertis *et al.* 1984) which in turn activate PKC by liberating DAG and AA from mucosal phospholipids, respectively (Figure 3, Reddy *et al.* 1996). Colonic bacteria are known to exhibit PLC activity (Reddy *et al.* 1996) and may therefore contribute to luminal DAG production. It has also been suggested that specific fatty acids in red meat, such as palmitic acid (C16:0), may lead to the formation of highly mitogenic DAG species (Friedman *et al.* 1989, Giovannucci and Goldin 1997).

It has been demonstrated that both dietary fat and fibre affect the composition and amount of faecal DAG in rats (Pickering *et al.* 1995) and that fibre supplementation can reduce faecal DAG concentration in humans (Reddy *et al.* 1994). Neither of these studies determined whether these alterations in DAG were associated with mucosal PKC activity. In accordance with this hypothesis, Reddy and co-workers (1996) found that carcinogen-treated rats fed a high corn oil diet had higher levels of secondary bile acids and higher activities of bacterial 7α-dehydroxylase and PLC as well as a lower mucosal activity of membrane PKC than rats fed a high fish oil diet or a low fat control diet. In contrast, the study of Chapkin and co-workers (1993) showed an increase in membrane PKC activity and membrane:cytosol ratio in rats fed fish oil compared to corn oil-fed rats. The same study found no difference between the effects of pectin and cellulose on PKC activity. Furthermore, feeding a diet containing 5% corn oil and 15% beef tallow resulted in an increase in colon membrane PKC activity relative to a group fed a low corn oil diet (Lafave *et al.* 1994). More recent studies have focused on the effects of corn oil and fish oil on colonic PKC isozyme expression (Davidson *et al.* 1995, Jiang *et al.* 1997a). The results of these studies suggest that the protective effect of fish oil against colon cancer may be partly mediated through fish-oil induced changes in expression of specific PKC isozymes during the carcinogenic process. Overall, relatively few studies have examined the effects of diet on colonic PKC activity and isozyme expression, and because of the mixed results only limited conclusions can be made.

4.1.2 Prostaglandin production

An extensively studied mechanistic hypothesis has been that dietary fats affect colon carcinogenesis through modulating the production of prostaglandins and other eicosanoids in the colonic mucosa. This hypothesis is supported by a number of human and animal studies showing that NSAIDs, inhibitors of COX enzymes, reduce both colonic prostaglandin biosynthesis and tumourigenesis. Favourable dietary fat composition is thought to modulate phospholipid fatty acid composition of mucosal cell membranes so that less AA would be available for prostaglandin biosynthesis. This appears to be true for fish oil. For example, supplementation of healthy volunteers with fish oil providing 4.4 g n-3 fatty acids per day for four weeks resulted in decreased proliferation and PGE_2 release from rectal biopsy specimens (Bartram *et al.* 1993). In rats, feeding diet high in fish oil led to significantly lower AA content of mucosal phospholipids

and lower PGE production when compared to feeding diets high in corn oil or beef tallow (Lee *et al.* 1993). A recent study with carcinogen-treated rats demonstrated that rats fed fish oil had decreased COX-2 expression as well as decreased tumour multiplicity and incidence when compared with rats fed corn oil (Singh *et al.* 1997). Furthermore, supplementation with EPA resulted in significant reductions in the AA content of phospholipids, PGE_2 formation, and tumour number in the intestine of Min mice (Hansen Petrik *et al.* 2000). These results confirm that when dietary manipulation is able to suppress colonic prostaglandin production, it is also likely to inhibit tumourigenesis. However, apart from large amounts of fish oil, there have been no significant differences between the effects of saturated fats, mainly from animal origin, and unsaturated fats, mainly vegetable oils with high linoleic acid content, on colonic AA levels and prostaglandin production (Lee *et al.* 1993). Administration of pure AA at low to moderate amounts has been shown to lead to its effective incorporation into tissue phospholipids and enhanced prostaglandin production in experimental animals (Whelan *et al.* 1993). Therefore it is possible that dietary AA, contributed by red meat intake, might be able to modulate eicosanoid production to a significant extent.

4.2 Mechanisms related to red meat

Heterocyclic amines

A much-favoured hypothesis is that the promotive effect of red meat on colon cancer can be explained by heterocyclic amines (HCA) that are formed during high-temperature cooking of meat. HCAs are mutagenic (Wakabayashi *et al.* 1992) and PhIP (2-amino-1-methyl-6-phenylimidatzo[4,5-*b*]-pyridine), the most abundant HCA in well-cooked meats, has been shown to induce intestinal tumours in rats (Ito *et al.* 1991) and Min mice (Steffensen *et al.* 1997, Paulsen *et al.* 1999). Furthermore, a recent study of Pence and co-workers (1998) demonstrated that the feeding of a beef diet containing high levels of HCA enhanced colon tumour formation in dimethylhydratzine-treated rats when compared with the corresponding diet low in HCAs, although this was seen only in the low-fat context. A positive relation between cooking processes and colon cancer risk has also been observed in some epidemiologic studies. A Swedish case-control study found an elevated colon cancer risk among those meat eaters who frequently

consumed brown gravy and preferred meat with a heavily browned surface (Gerhardsson de Verdier *et al.* 1991).

The cancer risk related to HCAs may also depend on the extent to which HCAs are activated in the body. Cytochrome P4501A2 and N-acetyltransferase-1 and -2 are responsible for the metabolic activation of HCAs and all the three enzymes are known to be polymorphic in humans. The N-acetyltransferase genotype, in particular, may be associated with colon cancer risk so that red meat eaters may be at higher risk of colorectal cancer if they are rapid acetylators (Chen *et al.* 1998).

N-nitroso compounds

One possibility is that diets high in red meat lead to increased protein fermentation within the colon, resulting in enhanced formation of endogenous carcinogens and tumour promoters. The amount of protein in the diet is a primary determinant of the amount of nitrogen, mainly in the form of protein, peptides, and amino acids, entering the colon (Silvester and Cummings 1995). These nitrogen sources could be fermented by colonic bacteria to potentially harmful or toxic substances, such as ammonia and *N*-nitroso compounds. High luminal ammonia concentrations have been associated with increased cell proliferation (Lin and Visek 1991a and 1991b), and ammonia has been shown to promote colon carcinogenesis in the rat (Clinton *et al.* 1988). Luminal *N*-nitroso compounds may be an important source of DNA-damaging alkylating agents which may produce base substitutions characteristic of those found commonly in colon tumours (Saffhill *et al.* 1985, Jacoby *et al.* 1993). In human volunteers, high red meat consumption (600g per day) led to increased plasma urea and faecal ammonia levels, and 3-fold increase in faecal N-nitroso compounds, whereas consumption of 600 g white meat and fish had no such effect (Bingham *et al.* 1996, Silvester *et al.* 1997). The authors concluded that the increase in faecal *N*-nitroso compounds is brought about by a specific effect of red meat and may be partly due to heme iron being a catalyst for *N*-nitroso compound formation.

Iron

The high iron content of red meat may also contribute to colon carcinogenesis. It has been suggested that iron increases colon cancer risk either by elevating faecal iron (Babbs 1990) or by

elevating tissue iron (Toyokuni 1996). High amounts of unabsorbed faecal iron may lead to enhanced generation of free radicals at the mucosal surface of the colon and thus to increased lipid peroxidation and DNA damage. In addition, iron may catalyse the formation of tumour promoters or the conversion of pro-carcinogen to carcinogen within the lumen of the colon (Babbs 1990). Excess plasma iron in the form of iron-transferrin may stimulate mucosal cell proliferation by providing biologically available iron for cell growth. Alternatively, iron overload could induce oxidative stress leading to tissue damage (Toyokuni 1996).

In line with these hypotheses, high dietary iron was shown to increase crypt cell proliferation and tumour development in the colon of carcinogen-treated rats (Nelson *et al.* 1989). However, dietary iron at a level more representative of human intake had no effect on cell proliferation, lipid peroxidation, or ACF development in AOM-treated rats (Soyars and Fischer 1998). Studies in humans have supported the positive association between body iron stores and colon cancer risk (Knekt *et al.* 1994a, Wurzelman *et al.* 1996). Knekt and co-workers (1994a) found an elevated risk of colon cancer in men and women with transferrin saturation over 60%. Another recent study reported a positive relationship between colon cancer and serum iron in women (Wurzelman *et al.* 1996).

4.3 Mechanisms related to fibre rich foods

Butyrate

In addition to the bulking effect and dilution capacity, dietary fibre may affect colon carcinogenesis through its fermentation products, such as butyrate, propionate, and acetate. Of these short-chain fatty acids, butyrate has been suggested to play an important role in colon cancer prevention. Butyrate is the principal energy source of the epithelial cells (Roediger 1982) and it also takes part in regulation of cell proliferation, differentiation, and apoptosis. The effects of butyrate on these cellular processes have often been opposite in normal colonocytes relative to neoplastic colonic cells. In normal colonocytes butyrate has been shown to increase cell proliferation (Sakata 1987, Lupton and Kurtz 1993), whereas in colon cancer cell lines butyrate has promoted expression of differentiation markers (Whitehead *et al.* 1986) and induced apoptosis (Hague *et al.* 1995). The growth inhibitory effect of butyrate in colon cancer cells may

be mediated by histone hyperacetylation and induction of $p21^{waf1}$, a cell cycle regulatory protein (Archer *et al.* 1998).

Experimental colon cancer studies partly support the protective effect of butyrate *in vivo*. McIntry and co-workers (1991) demonstrated that rats fed wheat bran maintained relatively high butyrate concentrations in the luminal contents along the entire length of their large intestine, particularly in the distal colon. In a further study they showed that wheat bran reduced colonic tumour development and that tumour mass was negatively correlated with faecal butyrate concentration in carcinogen-treated rats (McIntry *et al.* 1993). They concluded that the protective effect of wheat bran is due to its ability to maintain butyrate production in the distal colon where the majority of tumours occur. In contrast, Zoran and co-workers (1997) compared the effects of oat bran and wheat bran in a rat colon cancer model and found no relationship between tumour formation and high butyrate concentrations in the distal colon. Rats fed oat bran had significantly higher butyrate concentrations in both the proximal and distal colon but also significantly more tumours than wheat bran-fed rats.

Antioxidants and phytoestrogens

In addition to the fibre content, fibre rich foods contain a number of other substances that may have cancer preventive effects. Whole grains contain vitamins, trace minerals, and phenolic compounds such as phytic acids and phytoestrogens. Of these compounds, vitamin E, selenium, phenolic acids, and phytate have been proposed to function as antioxidants, protecting cells from oxidative damage (Slavin *et al.* 1999). Phenolic compounds may also induce the detoxification systems, specifically the phase II conjugation reactions.

Whole grains, especially rye and wheat, are rich sources of the plant lignans, so called phytoestrogens, which are converted by gut bacteria to the mammalian lignans enterolactone and enterodiol (Adlercreutz and Mazur 1997). These phytoestrogens have been proposed to bind weakly to the oestrogen receptor and therefore they may have either a mild oestrogen-like action or they may antagonise oestrogen action. Phytoestrogens may have relevance in colon cancer prevention because age-related hypermethylation of the oestrogen receptor gene is currently thought to be one of the molecular pathways leading to colon cancer (Issa *et al.* 1994). In colon

cancer cells, hypermethylation results in inactivation of the oestrogen receptor gene, which is accompanied by deregulated growth (Issa *et al.* 1994). Circulating oestrogens have been shown to modulate the expression of oestrogen receptor in several tissues (Tata *et al.* 1993), suggesting that blood levels of oestrogens or phytoestrogens might be able to attenuate the inactivating effect of hypermethylation on the oestrogen receptor gene. This hypothesis is supported by some epidemiological studies which have shown that postmenopausal oestrogen replacement is associated with a reduced risk of colon cancer (reviewed in Giovannucci and Platz 1999). Furthermore, in carcinogen-treated rats feeding phytoestrogen sources, such as flaxseed or lignan precursor, inhibits ACF formation in the colon (Serraino and Thompson 1992, Jenab and Thompson 1996). However, it is not yet clear whether these tumour preventive effects of phytoestrogens are really oestrogen receptor mediated since these compounds have not always shown oestrogenic or antioestrogenic activity or response though they have been effective in inhibiting tumour growth (Saarinen *et al.* 2000).

5. OBJECTIVES OF THE STUDY

Diet is considered to be one of the most important environmental contributors to the development of colon cancer. Based on epidemiological findings, the main three dietary factors thought to modulate colon carcinogenesis are fat, red meat, and fibre rich foods. A number of epidemiological and experimental studies have tried to determine the relationship between these dietary factors and colonic tumour formation but the results have been more or less controversial. Therefore, studies elucidating the mechanisms by which diet may modulate colon tumourigenesis would help in setting future dietary recommendations. This dissertation summarises the results of six studies which were carried out to determine the effects of fat, red meat, and different fibre sources on cell signal transduction molecules, mainly on PKC, in the colonic mucosa of healthy rats and on tumour development in the intestine of Min mice.

This dissertation specifically aims to answer the following questions:

1. Do type and amount of dietary fat, red meat (beef) or different fibre sources affect steady-state PKC activity and isozyme expression in the colonic mucosa of rats (I, II, III, IV, V)?
2. Are these effects mediated by changes in mucosal phospholipid fatty acid composition and/or faecal DAG (II, III, V)?
3. Does beef affect colonic sphingomyelinase activities (III, IV)?
4. Are the effects of beef related to its arachidonic acid content (III)?
5. Do dietary fat, beef, and different fibre sources modulate intestinal tumour development in Min mice (VI)?
6. Could the dietary effects on tumour development be explained by changes in PKC and β-catenin (VI)?

6. STUDY DESIGNS AND METHODS

6.1 Study designs and diets

Studies I and II

These two studies were carried out to determine whether type and amount of dietary fat affect steady-state PKC activity in rat colonic mucosa. In Study I, rats were fed one of the three high-fat diets (43% energy from fat) containing either butter (high in saturated fatty acids), rapeseed oil (high in n-9 fatty acids), or sunflower seed oil (high in n-6 fatty acids) as a fat source. The control group was fed a low-fat (10% of energy) sunflower-seed-oil diet. Total PKC activity and fatty acid composition of the colonic mucosa were analysed after a four-week feeding period.

Because the results of Study I indicated that a high-fat butter diet increases colonic PKC activity, Study II was done to see whether this observation is typical of saturated fats in general or specific for fats rich in certain types of saturated fatty acids, i.e. palmitic (16:0) and stearic (18:0) acids. Therefore, the effects of two different saturated fats, butter and coconut oil, were compared in Study II. Two levels of the fats (10% and 43% of energy) were used to study the effect of fat amount on PKC activity. Because dietary fat is likely to affect colonic PKC activity by modulating PKC activators in luminal contents and/or mucosal cells, faecal DAG concentration and mucosal phospholipid fatty acid composition were analysed.

Studies III and IV

Study III consisted of two experiments referred to as IIIa and IIIb. Study IIIa was done to compare the effects of two protein sources, casein and beef, on colonic PKC activity and faecal DAG concentration in rats. To ensure the same protein concentration of the AIN93G-based diets (i.e. 20% by weight, Reeves *et al.* 1993), the beef was dried to constant moisture and ground before addition to the diet. Apart from the protein composition, the beef diet differed from the casein diet in that it contained lipids of cell membranes and a small amount of AA.

Study IIIa showed that the beef diet induced PKC activation in the colonic mucosa and therefore Study IIIb was carried out to determine whether this effect was due to the small but possibly

biological active amount of AA present in the beef diet. Because fish oil fatty acids are known to antagonise several cellular functions induced by AA, a fish oil-supplemented group was also included in the study. Rats were fed an AIN-93G basal diet which was supplemented with a daily dose of 300 ml soybean oil (control group), or 8 mg of AA in 300 ml soybean oil, or a mixture of 99 mg of EPA and 67 mg of DHA. The supplements were given by syringe to ensure that each rat received the proper dose. Mucosal PKC activity, PGE_2 concentration, activities of acid, neutral and alkaline sphingomyelinases, and fatty acid composition of phospholipids were analysed after the supplementation period of four weeks.

Study IV was done to extend the findings of Study IIIa by determining the effects of a high-beef diet on PKC isozyme expression. The PKC isozymes analysed were PKC α, β2, δ, ζ/λ, and ζ. Sphingomyelinase activities were also analysed.

Study V

Study V was designed to test whether different fibre sources are able to modulate PKC activity and isozyme expression in rat colonic mucosa and if so, whether these effects are mediated by changes in luminal DAG levels. Rats were fed one of the following AIN93G-based diets: a non-fibre high-fat diet or one of the four high-fat diets supplemented with either rye, oat or wheat bran or inulin at 100 g/kg diet. As the non-fibre diet was designed to approximate an average human Western type diet its fat content was high, i.e. 40% of energy, and the ratio of saturated:monounsaturated:polyunsaturated fatty acids was approximately 3:2:1 of the energy given by fat. PKC activity was analysed in the proximal and distal colon separately and the level of DAG in the contents of distal colon.

Study VI

Study VI was carried out to determine the effects of fat, red meat, and different fibre sources on intestinal tumour development in Min mice. The mice were divided into seven diet groups: 1) AIN93G diet with low fat content and 5% (w/w) of cellulose as a fibre source, 2) high-fat non-fibre diet (the same as in Study V), 3-6) high-fat diets supplemented with 10% (w/w) of wheat, rye, or oat brans or 2.5% (w/w) of inulin, 7) high-fat non-fibre diet with beef as a protein source. After the feeding period of five to six weeks, the number and size of tumours in the small and

large intestine were determined and mucosal levels of PKC isozymes and β-catenin were analysed.

6.2 Animals and sample collection

The study protocols for all the experiments were approved by the Laboratory Animal Ethics Committee of the Faculty of Agriculture and Forestry, University of Helsinki. Male weanling Wistar rats were obtained from the National Laboratory Animal Centre, University of Kuopio, Finland, for Studies I and II, from the Experimental Animal Facility of University of Helsinki, Finland, for Studies III and IV, and from Harlan Co., The Netherlands, for Study V. Male Min mice (4-7 weeks of age) for Study VI were obtained from the Jackson Laboratory, ME, USA. During the experiments, rats and Min mice were housed in plastic cages in an animal facility with a controlled temperature (20-22°C) and a 12 h light-dark cycle. During the experiments rats and mice had free access to the diets and tap water. Body weights were recorded weekly. The number of animals per dietary treatment group was 12 - 13 in the studies using rats and 7 - 9 in the study using mice. In studies II, III, and V, each rat was kept in a metabolic cage for 1 to 2 d to collect faeces. At the end of the feeding periods the animals were killed by CO_2 asphyxiation.

In Studies II – V rat colons were removed, cut open longitudinally, and flushed clean with ice-cold phosphate-buffered saline. The colons were divided into proximal and distal segments of equal length; mucosas were scraped off with a microscope slide and used for biochemical analysis. Study I differed from the other studies in that the proximal one-thirds of the colonic mucosa was used for fatty acid analysis and the distal two-thirds for PKC activity analysis. The caecal mucosa was used for phospholipid fatty acid analysis in Study II. It was collected essentially in the same way as the colonic mucosa. Those tissue and faecal samples that were not used for the analysis on the same day were frozen in liquid N_2 and stored at -70°C.

6.3 Methods

Tumour scoring in Study VI

The whole intestine of the Min mice in Study VI was removed, opened longitudinally, and flushed with ice-cold physiological saline. The small intestine was divided into five segments of equal length. The caecum and colon were kept together. The segments of the small intestine and the large intestine were then spread out, mucosal surface up, on microscope slides and the number, diameter, and location of tumours were determined using an inverse light microscope at a magnification of x 2.5. The mucosa of the distal small intestine was then scraped off, frozen in liquid N_2, and stored at -70°C until used for PKC isozyme and β-catenin analyses.

PKC activity and isozyme expression

Mucosal samples from rat colon (I-V) and mice small intestine (VI) were homogenised by sonication and ultracentrifuged at 100 000 *g* for 1 h. The supernatant was collected and used as the cytosolic fraction. The pellet was resuspended in the extraction buffer containing 0.2% (v/v) Triton X-100, incubated for 20 min and ultracentrifuged at 100 000 *g* for 1 h. The resulting supernatant contained the membranes (particulate fraction). Cytosolic and membrane fractions were further purified by DEAE-cellulose chromatography. PKC activity of the DEAE-purified cytosolic and membrane fractions was assayed using a modification of the method described by El Touny and co-workers (1990). In this method, PKCs activated by phosphatidylserine-diacylglycerol vesicles phosphorylate a lysine-rich histone protein (histone III-S, Sigma, USA) in the presence of [^{32}P]ATP. PKC activity is expressed as pmol of ^{32}P incorporated per min per mg protein. The enzyme activity measured without the activators comprised 6-10% of the total PKC activity. An addition of 50 μM myristoylated octapeptide (Bachem, Switzerland) to the reaction mixture inhibited enzyme activity by 87% and 94% in cytosolic and membrane fractions respectively. Protein concentrations of the crude and DEAE-purified fractions were measured using a Bio-Rad protein assay reagent based on the Bradford method (Bradford 1976) with bovine serum albumin as a standard.

For PKC isozyme analysis (IV, V, VI), 2 ml of the crude cytosolic and membrane extracts were concentrated to 1/20 volume with Centrex UF-2 concentrators (Schleicher & Schuell, Germany).

The samples were then mixed with an equal volume of sodium dodecylsulphate (SDS) sample buffer, boiled, and stored at -80^{o}C until use. A pooled rat brain homogenate, containing both cytosolic and membrane proteins, was used as a normalisation factor and a control for PKC antibody specificity in immunoblotting analysis. Rat brain homogenate was prepared essentially in the same way as described for the colon samples. Colonic mucosa samples (30-100 μg) and rat brain homogenate (1-5 μg) were subjected to SDS-polyacrylamide gel electrophoresis using 10% resolving gels (Laemmli 1970) and then transferred to polyvinylidene difluoride membranes (Towbin 1979). Following transfer, the membranes were probed with antibodies for PKC α, β2, δ, and ζ/λ by the method of Scheng and Schuster (1992), protocol B. Dilution of individual primary antibody and alkaline phosphatase-conjugated secondary antibody was optimised for each PKC isozyme. PKC bands were visualised by colorimetric staining of blots with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrate mix (Bio-Rad, CA, USA). Blots were scanned on a Sharp JX325 Scanner and the scanning images were analysed with ImageMaster 1D Software, version 2.0 (Pharmacia Biotech, Sweden). The results are expressed as sample band intensity divided by rat brain band intensity. In preliminary experiments, a range of protein concentrations for each isozyme was loaded onto gels to ensure that the colorimetric signal was quantitatively detectable.

Phospholipid fatty acid composition

Total lipids of caecal (II) and colonic mucosa (III) were extracted by the method of Folch and co-workers (1957). Phospholipids of colonic samples (III) were fractionated by thin layer chromatography (silica gel 60, Merck, Germany). Thereafter samples were methylated as described previously (Stoffel *et al.* 1959). The fatty acid methyl esters derived from total lipids or different phospholipid fractions were analysed using a Hewlett Packard 5890 Series II gas chromatograph (USA) equipped with a fused silica capillary column (NB-351, Nordion, Finland) and an ultraviolet detector. The oven temperature was programmed to rise from 170°C to 230°C at a rate of 4°C/min and then kept at 230°C for 8 min. Fatty acid peaks were identified by comparison with a fatty acid methyl ester standard (GLC-91, Nu-Chec-Prep, MN, USA). The proportions of individual fatty acids are expressed as percentages of the total fatty acids.

PGE_2

Colonic mucosal samples (III) were homogenised in 15% ethanol and centrifuged at 5000 *g*. The supernatant was removed and acidified to pH 3. The samples were then purified by SepPak-C18 cartridges (Waters Assoc., MA, USA) according to Powell (1982) and their PGE_2 content was determined by enzyme immunological assay using a commercial kit (Cayman Chemical, MI, USA). Prior to the purification procedure, 5000 cpm of $[^3H]PGE_2$ was added to the samples to assess recovery. Mucosal PGE_2 is expressed as ng per mg sample protein.

DAG concentration in luminal contents and faeces

Lipids from luminal contents (V) and faecal samples (II, III) were extracted by a modified method of Bligh and Dyer (1959). The lipid extracts were dried under nitrogen and used for analysing the total amount of DAG by a commercial kit (Amersham, UK) based on the *E. coli*-DAG-kinase method (Preiss *et al.* 1986). After the enzymatic reaction the samples were purified by Amprep Si -columns (Amersham, UK) according to the manufacturer's instructions and subjected to scintillation counting.

β-Catenin expression

Mucosal samples of the distal small intestine of Min mice (VI) were treated in the same way as samples for PKC isozyme analysis. The protein levels of β-catenin were determined in cytosolic and particulate fractions by using monoclonal mouse β-catenin antibody (Transduction, KY, USA) and alkaline phosphatase-conjugated anti-mouse secondary antibody (Zymed, CA, USA). Quantitation of β-catenin signal was as described for PKC isozymes.

Sphingomyelinase activities

Sphingomyelinase activities of colonic mucosal samples in Studies III and IV were kindly analysed by Dr. Rui-Dong Duan's group at the University Hospital of Lund, Sweden. Sphingomyelinases were determined as described previously (Hertevig *et al.* 1997) with modifications (Papers III and IV).

Statistical analysis

One-way analysis of variance was used to compare variables among the dietary groups in Studies I, II, IIIb, V, and VI. When the p value was < 0.05, means were separated using Tukey's *post hoc*-test. In Study IIIa, the unpaired t-test was used to compare the means between the beef and casein groups. The differences between the proximal and distal colon were analysed by the paired samples t-test. The correlation data were analysed by either the Pearson correlation analysis or a linear regression analysis. The Systat statistical computer package (version 7.0, SPSS Inc., USA) was utilised for all the statistical analyses.

7. RESULTS

7.1 Effects of fat, red meat, and fibre sources on colon signal transduction (PKC) in rats (I-V)

Fat (I-II)

In Study I the high-fat butter diet resulted in increased membrane PKC activity in rat colonic mucosa when compared with the low-fat control diet ($p < 0.05$; Figure 4a). PKC activity in rats fed the high-fat rapeseed and sunflowerseed-oil diets was close to that in the control group. In Study II we were unable to reproduce the increasing effect of butter on PKC activity though membrane PKC activity in the distal colon appeared to be slightly higher in rats fed the butter diets than in those fed the control diet (Figure 4b). There were also no dietary effects on PKC activity in the proximal colon (Table 3 in Paper II). The effects of the coconut-oil diets on PKC activity were essentially similar to those of the butter diets. The saturated fat diets caused relatively small changes in fatty acid composition of caecal phospholipids, with rats fed the high-fat coconut-oil diet having an increase in 14:0 and rats fed the high-fat butter diet having an increase in 18:1*n*-9 when compared to the rats in the control group (Tables 5 and 6 in Paper II). The fatty acid composition of phosphatidylcholine was associated with membrane PKC activity so that saturated fatty acids (14:0 and 18:0) were positive correlated and unsaturated fatty acids (e.g. linolic acid, AA) were negatively correlated with membrane PKC activity (Figure 1 in Paper II). Faecal DAG concentration was not significantly affected by the diets (Figure 4c).

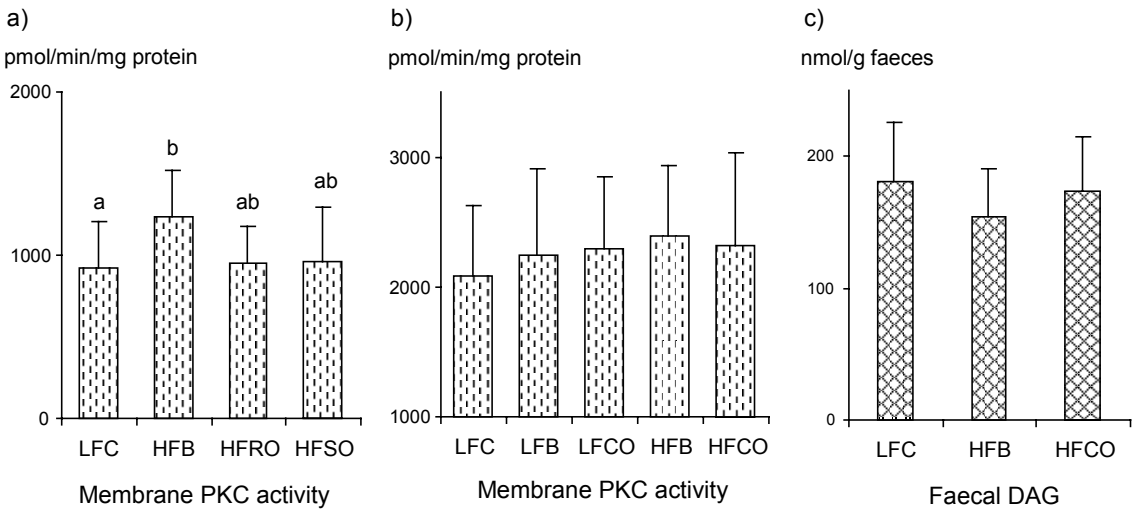

Figure 4. Effects of dietary fats on a) membrane PKC activity in whole colon mucosa, b) membrane PKC activity in distal colon mucosa, and c) faecal DAG concentrations in rats. Values are means and SD. Bars with different letters are significantly different ($p < 0.05$). LFC, low fat control, HFB, high fat butter, HFRO, high fat rapeseed oil, HFSO, high fat sunflower seed oil, LFB, low fat butter, LFCO, low fat coconut oil, HFCO, high fat coconut oil.

Red meat and arachidonic acid (III-IV)

Rats fed the beef diet had significantly higher membrane and cytosolic PKC activities in their distal colons than rats fed the casein diet ($p < 0.05$ and $p < 0.01$ respectively; Figure 5a). The beef diet induced PKC activity also in the proximal colon, though the effect was less pronounced than in the distal colon (Table 3 in Paper III). Changes in distal PKC isozyme expression occurred, with beef-fed rats having a higher level of cytosolic PKC α and a lower level of membrane PKC δ than casein-fed rats (Figure 2 in paper IV). PKC ζ/λ was affected so that the level of 40 & 43 kDa fraction was higher and the level of 70 & 75 kDa fraction was lower in beef-fed rats (Figure 5b and 5c). There was no significant difference in faecal DAG concentrations between the beef and casein groups (169 ± 33 vs. 158 ± 19 nmol/g wet faeces). DAG concentration was positively correlated with the cytosolic PKC activity (Figure 1 in Paper III).

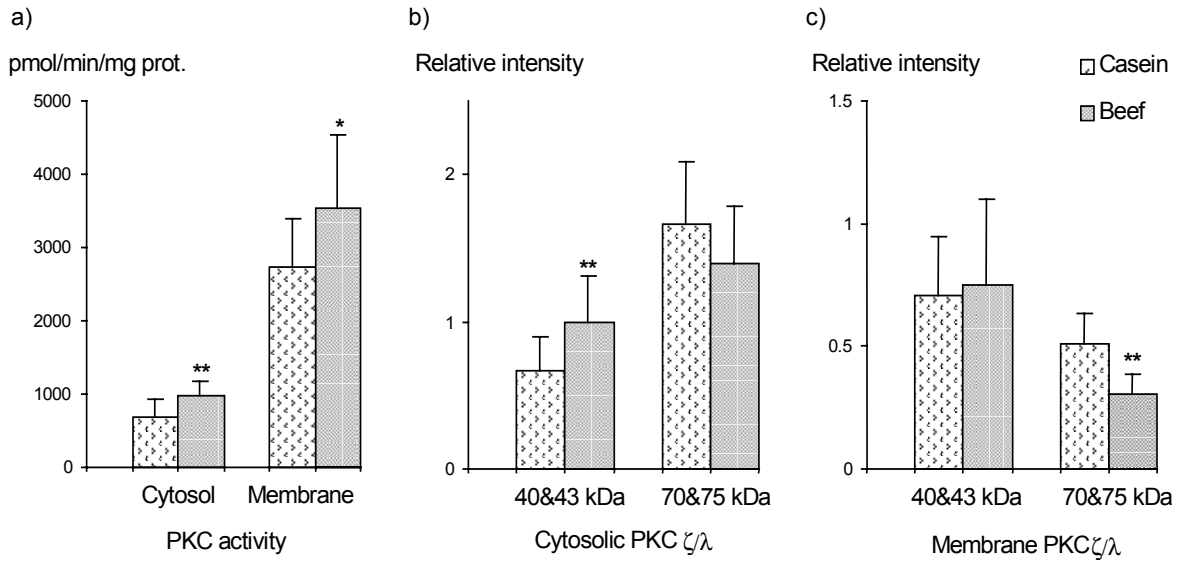

*Figure 5. Effects of the beef diet on a) PKC activity, and PKC ζ/λ protein levels b) in the cytosol and c) in the membrane of rat distal colon. Values are means and SD. * and **, significantly different from the casein group at $p < 0.05$ and $p < 0.01$, respectively.*

Supplementation of AA with an amount equivalent to that available from the beef diet had no effect on PKC activity in rat distal colonic mucosa. However, AA-supplemented rats had a significant ($p < 0.05$) increase in proportion of AA in mucosal phosphatidylcholine compared with the AIN93G control diet (Table 5 in Paper III). This was accompanied by an increase in concentration of mucosal PGE_2, which was significant only in comparison with the fish oil supplemented group (Table 6 in Paper III). Neither beef nor AA and fish oil supplementations affected the activities of acid, neutral, and alkaline sphingomyelinase (Table 6 in Paper III and Table 3 in Paper IV).

Fibre sources (V)

In the distal colon, wheat bran feeding resulted in the lowest mucosal PKC activity and PKC β2 protein level, whereas oat bran and inulin feeding led to an increase in PKC activity and PKC β2 levels (Figure 6a and 6b). Rats fed the non-fibre control diet were between these two extremes. Rye bran-fed rats had low PKC activity although their PKC β2 expression was rather increased than decreased. DAG concentration in the contents of distal colon was the lowest in the non-fibre and wheat bran groups which differed significantly ($p < 0.05$) from the rye bran group (Figure

6c). The increasing effect of inulin on membrane and total PKC activity was even more pronounced in the proximal than in the distal colon (Table 5 in Paper V). However, isozyme expression differed so that, instead of PKC β2, inulin induced membrane PKC δ expression (Table 3 in Paper V). There were also changes in expression of cytosolic PKC α and ζ/λ in the proximal colon and cytosolic PKC δ and ζ/λ in the distal colon (Table 3 and 4 in Paper V).

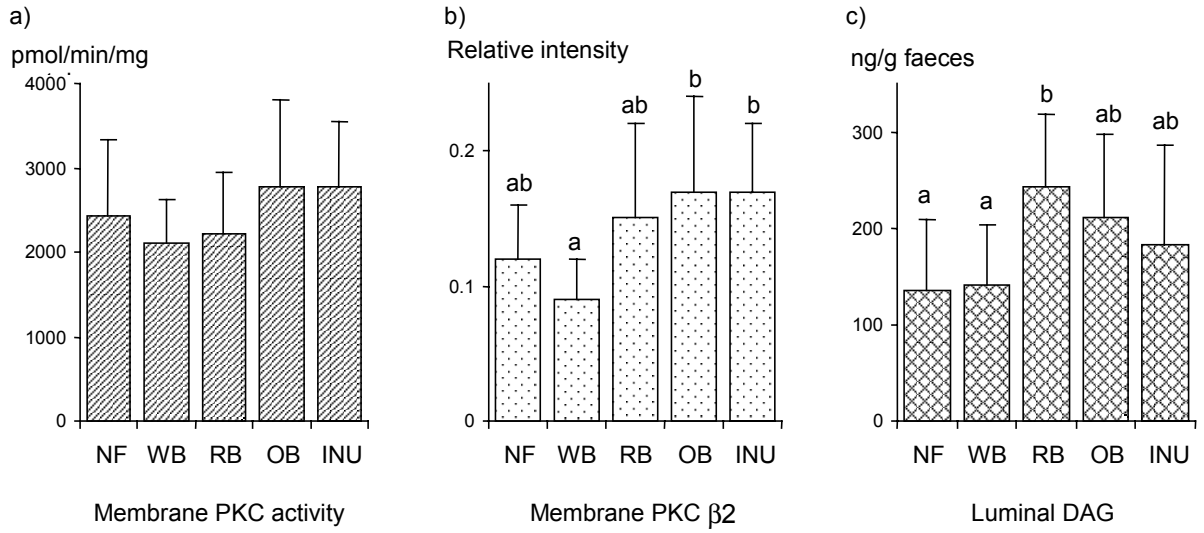

Figure 6. Effects of diets enriched with different fibre sources on a) membrane PKC activity and b) membrane PKC β2 protein levels in rat distal colon mucosa as well as c) DAG concentrations in the distal colon contents. Values are means and SD. Bars with different letters are significantly different ($p < 0.05$). NF, non-fibre high-fat, WB, wheat bran, RB, rye bran, OB, oat bran, INU, inulin.

7.2 Effects of fat, red meat, and fibre sources on intestinal tumour development in Min mice (VI)

In Min mice, the majority of tumours develop in the distal small intestine. The number of tumours in the distal small intestine was lowest in mice fed the rye bran diet and highest in mice fed the beef diet. The beef-fed mice differed significantly ($p < 0.05$) from mice fed the rye bran, wheat bran, and AIN93G diets (Figure 7a). The inulin-fed mice had nearly as many tumours as the beef-fed mice and they differed significantly from the mice fed the rye bran diet (Figure 7a).

Though Min mice have only few tumours in their colons, the effects of the experimental diets on colonic tumour development were in line with those seen in the small intestine (Table II in Paper VI). Specifically, tumour incidence was only 33% in the rye bran group when compared with 89% in the beef group and 100% in the inulin group. There were no significant differences in tumour size among the diet groups (Table III in Paper VI).

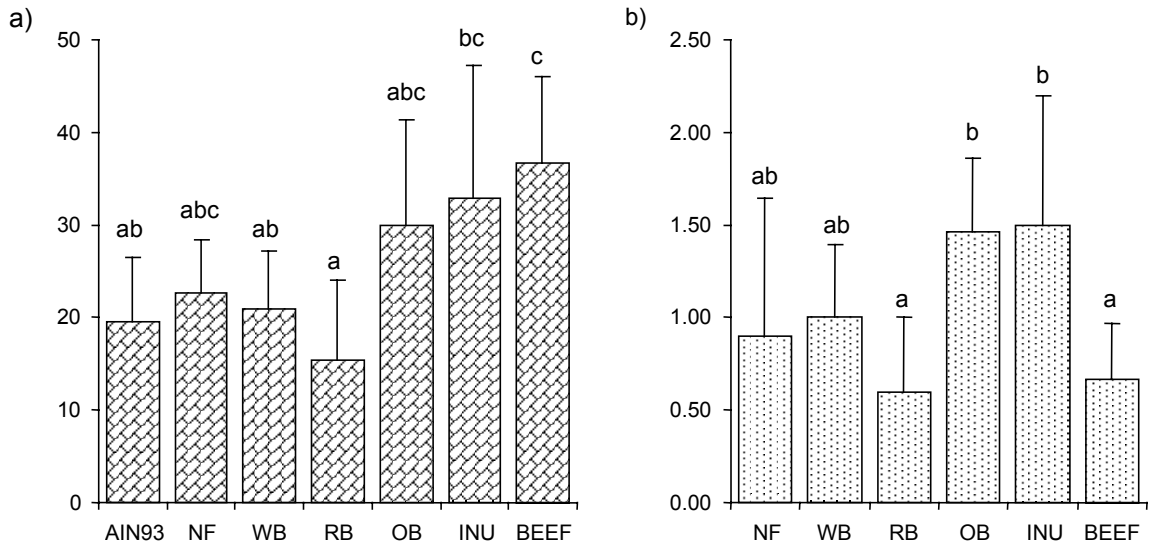

Figure 7. Effects of fat, beef, and fibre sources on a) intestinal tumour formation and b) mucosal β-catenin protein level in Min mice. Values are means and SD. Bars with different letters are significantly different ($p < 0.05$). NF, non-fibre high-fat, WB, wheat bran, RB, rye bran, OB, oat bran, INU, inulin.

In general, the protein levels of PKC α, β2, δ, and ζ in the distal small intestinal mucosa of Min mice were much higher in the cytosolic than in the membrane fraction. Membrane levels of PKC α and δ were under the detection limit. There were large variations in PKC values within dietary groups and no significant differences in PKC isozyme expression between the dietary groups could be found (Table IV in Paper VI). Cytosolic β-catenin level was lowest in mice fed the rye bran and beef diets and highest in mice fed the inulin and oat bran diets (Figure 7b). The inulin group differed significantly ($p < 0.05$) from the non-fibre, rye bran, and beef groups, and the oat bran group differed from the rye bran and beef groups. Cytosolic β-catenin level was significantly correlated with all the PKC isozymes analysed ($r = 0.62 – 0.68$, $p < 0.001$).

8. DISCUSSION

8.1. Fat

Dietary fat appeared to have no major role in modulating PKC activity in the colonic mucosa of rats. The first study showed some indication that high-fat butter diet, rich in saturated fatty acids, increases membrane PKC activity when compared to a low-fat basal diet and, to a lesser extent, to high-fat rapeseed and sunflower-oil diets, rich in unsaturated n-9- and n-6-fatty acids, respectively. The purpose of the second study was to determine further the role of saturated fats in PKC activation but what could be seen was only a slight increasing effect of saturated fat on PKC activity in the distal colon. There were also no difference between the effects of butter and coconut oil, demonstrating that chain length of saturated fatty acids is not important (palmitic and stearic acids vs. lauric and myristic acids). Results of two previous studies with rats indicated an increase in membrane PKC activity by feeding beef tallow relative to feeding corn oil (Chapkin *et al.* 1993) or low-fat basal diet (Lafave *et al.* 1994).

The high-fat saturated diets had no effect on faecal DAG levels nor was there any correlation between DAG and mucosal PKC activity. This result contradicts the assumption that high-fat diets result in increased levels of faecal lipids and bile acids, which together enhance DAG production by colonic bacteria (Morotomi *et al.* 1990 and 1991). The DAG produced could then enter colonic epithelial cells and activate PKC, thereby causing a chronic state of increased cell proliferation. Though this hypothesis sounds plausible it has actually never been verified in *in vivo* studies.

The effects of butter and coconut oil on fatty acid composition of PC and PE fractions in caecal mucosa were rather moderate and no dietary effects were observed, for example in the levels of AA, a fatty acid considered to be important for signal transduction. The composition of PC was more resistant to dietary induced changes than that of PE. This may partly account for the relatively week effect of the fats on PKC activity, since PC hydrolysis products, DAG in particular, are thought to maintain the sustained PKC activity needed for long-term cellular responses in cells (Nishizuka 1995). Supporting the role of PC in regulating PKC activity, PC

fatty acids were significantly correlated with membrane PKC activity. Generally, saturated fatty acids were associated with increased and unsaturated fatty acids with decreased PKC activity. It seems unlikely that these associations were mediated through PC hydrolysis products since saturated DAG and saturated fatty acids have been week stimulators of PKC activity *in vitro* (Hannun *et al.* 1986). Alternatively, PC as the main phospholipid in cell membranes affects cell membrane physicochemical properties, which may have direct effects on PKC activity. This is supported by a study of Slatter and co-workers (1994) that showed with artificial membranes that increasing PC unsaturation in the presence of PE resulted in decreased PKC activity.

We could also see no promoting effect of a high-fat diet on intestinal tumour development in Min mice. The adenoma number in mice fed the high-fat diet, the fat being mostly butter, was nearly identical with mice fed the low-fat AIN93G diet. High-fat diets are generally considered to increase colon cancer risk and especially beef tallow and corn oil have promoted colon carcinogenesis in some (Reddy 1992, Wasan *et al.* 1997) but not all (Nauss *et al.* 1983) experimental colon cancer studies. However, the promotive effect of fat may be partly explained by its contribution to energy intake, since recent epidemiological studies (Howe *et al.* 1997) have shown no independent effect of fat on colon cancer risk after adjustment for total energy intake.

8.2 Red meat

Beef feeding resulted in a significantly increased PKC activity both in the cytosolic and membrane fractions of the distal colon of rats. The isozyme analysis gave no clear answer to the question of which isozyme is responsible for the increased PKC activity. Cytosolic PKC α was elevated in beef-fed rats compared with the casein-fed rats, but because PKC α requires DAG and Ca^{2+} and presumably the membrane environment for activation (Newton 1995), it is not likely to be active in the cytosol. In the membrane fraction, rats fed the beef diet showed no clear increase in any of the isozymes measured. Therefore the changes seen in cytosolic and membrane PKC ζ/λ expression, with beef-fed rats with a decrease in the 70 & 75 kDa fraction and an increase in the 40 & 43 kDa fraction, may have accounted for the increased PKC activity. Cellular fractions typically contain several immunoreactive bands of PKC ζ/λ (Davidson *et al.* 1994), probably as intermediate products of down-regulation (Yong *et al.* 1987). It has been

suggested that the low molecular bands are proteolytical products of PKC that lack the regulatory domain and are thus permanently active (Berra *et al.* 1993). Down-regulation of PKC ζ and λ is a feature of carcinogen-induced colonic tumours in rats (Jiang *et al.* 1997a, Wali *et al.* 1995, Jiang *et al.* 1997b, Roy *et al.* 1995), which has been blocked by feeding fish oil (Jiang *et al.* 1997a), a 1,25-dihydroxyvitamin D_3 analogue (Wali *et al.* 1996) or a nonsteroidal anti-inflammatory drug piroxicam (Roy *et al.* 1995). In line with epidemiological evidence, each of these agents was also able to reduce colonic tumour formation.

Beef feeding did not alter luminal DAG levels, suggesting that the increased PKC activity in beef-fed rats was not due to enhanced production of luminal DAG. Nevertheless, faecal DAG was positively correlated with the cytosolic PKC activity, which leaves the possibility that more DAG might have been produced and also taken up by colonocytes in beef-fed rats. DAG is assumed to enhance the translocation of PKC to the cell membrane (Newton 1995) and therefore the correlation of DAG to the cytosolic instead of membrane PKC activity was unexpected. However, as has been shown to occur in fibroblasts, DAG taken up by cells may also have been broken down to monoacylglycerol and fatty acids (Florin-Christensen *et al.* 1992). Based on *in vitro* studies, *cis*-unsaturated fatty acids are capable of selectively activating cytosolic PKC (Khan *et al.* 1991). Furthermore, DAG fatty acid compositions may have differed in the dietary groups. Though beef tallow was added to the casein diet to achieve similar fat concentrations and fatty acid compositions for the diets, the beef diet contained a small amount of AA, which was not present in the casein diet. Therefore the beef-fed rats may have had faecal DAG species containing more AA and being more effective in activating cytosolic PKC.

Because AA might have contributed to PKC activity by modulating not just DAG but phospholipid fatty acid composition, it was interesting to test whether the AA content of the beef diet was the reason for the increased PKC activity. This was, however, not the case since supplementation of AA at a level equal to that available from the beef diet caused no changes in mucosal PKC activity even though AA supplementation did increase the AA content of the PC fraction. In contrast to this result, a short-term exposure (10-min) of colon epithelium to free AA caused an increase in mucosal PKC activity in rats (Craven and DeRubertis 1988). However, it is likely that long-term effects of dietary AA differ from those of a short-term exposure. A fish oil

supplemented group was also included in the study because fish oil fatty acids are considered to antagonise several cellular functions induced by AA (Li *et al.* 1994). Fish oil supplementation had no effect on PKC activity. The results from previous *in vivo* studies with fish oil have been controversial, and both a decrease (Reddy *et al.* 1996) and an increase (Chapkin *et al.* 1993) in membrane PKC activity compared with corn oil feeding have been reported. It should be noted that the amount of fish oil given to rats in our study was considerably less than that in the previous studies. Also, there is evidence that large amounts of fish oil do alter PKC isozyme expression and that these changes are associated with inhibition of the carcinogenic process in rat colon (Jiang *et al.* 1997a and 1997b).

Because AA is the major precursor of eicosanoids and may affect sphingolipid metabolism, both of which are proposed to be involved in colon carcinogenesis, we also analysed mucosal PGE_2 concentration and sphingomyelinase activities. It is of interest that AA supplementation resulted in an increase in mucosal PGE_2, indicating that diets high in beef might modulate intestinal eicosanoid production. This is important considering the apparently significant role of COX-2 in colon carcinogenesis (Williams *et al.* 1997). Fish oil supplementation, on the other hand, caused a decrease in PGE_2 concentration, which has been demonstrated in previous studies (Bartram *et al.* 1993, Lee *et al.* 1993). Neither AA nor fish oil supplementation affected mucosal sphingomyelinase activities, suggesting that mucosal ceramide production and thus the growth suppressive pathways induced by ceramide were not altered. Apart from AA, beef contains other cell membrane components, such as sphingomyelin (Blank *et al.* 1992), which may modulate sphingomyelin activity. Therefore it was later checked whether the beef-fed rats had changes in their colon sphingomyelin activities. However, activities of acid, neutral, and alkaline sphingomyelinases were not different for the beef and casein-fed rats. Interestingly, acid sphingomyelinase activity was significantly correlated with both PKC activity and PKC ζ protein levels, which is in line with the previous *in vitro* studies showing that ceramide produced by acid sphingomyelinase activates PKC ζ (Lozano *et al.* 1994, Müller *et al.* 1995).

Consistent with the increase in PKC activity, the beef diet induced intestinal tumour development in Min mice. The promoting effect on tumourigenesis could be seen in both the small intestine and the colon. This result is in line with the recent prospective epidemiological studies that have

shown a positive association between red meat intake and colon cancer risk (Willet *et al.* 1990, Giovannucci *et al.* 1994). Experimental evidence concerning beef or red meat is limited and inconsistent in nature, with results showing both promotive and preventive effects of red meat on colon carcinogenesis (Nutter *et al.* 1983 and 1990, McIntosh *et al.* 1995). The discrepancy in results of the earlier studies is likely to be due to differences in other components of diets and in the form in which meat is given to animals. It is interesting that the beef-fed mice had no increase in their cytosolic β-catenin levels since elevation of cytosolic free β-catenin is considered to be the immediate reason for abnormal cell growth and thus for tumourigenesis in Min mice (Korinek *et al.* 1997). It is possible that beef feeding enhanced tumour formation by affecting cellular targets down-stream of β-catenin.

8.3 Fibre Sources

Feeding diets enriched with cereal brans or inulin modulated both PKC activity and isozyme expression in the rat colon. Overall, PKC activity in rats fed the wheat and rye bran diets was at the same level or lower than in rats fed the non-fibre diet, whereas rats fed the inulin and oat bran diets had increased PKC activity. In the distal colon, these changes in PKC activity were accompanied by changes in protein levels of membrane PKC β2, indicating activation of this particular isozyme in the inulin and oat bran groups. There is considerable evidence to suggest that PKC β2 is involved in colon cell proliferation and colon carcinogenesis. In colon cancer cell lines, PKC β2 has been directly linked to cell proliferation (Sauma *et al.* 1996, Sauma and Friedman 1996), which is supported by studies revealing a distinct pathway through which PKC β2 promotes mitosis in human leukemia cells (Gökmen-Polar and Fields 1998). In an *in vivo*-study with transgenic mice, overexpression of PKC β2 led to hyperproliferation of colonic epithelium and to an increased susceptibility for the formation of carcinogen-induced ACF (Murray *et al.* 1999). Furthermore, PKC β2 expression and/or membrane association are increased in colonic atypical crypts (Kahl-Rainer *et al.* 1996) as well as in tumours compared to the surrounding normal mucosa (Craven and DeRubertis 1992, Davidson *et al.* 1994, Wali *et al.* 1995), indicating that PKC β2 may enhance malignant growth.

The increasing effect of inulin on membrane PKC activity was even more pronounced in the proximal than distal colon. However, protein level of membrane PKC δ, not PKC β2, was increased in the proximal colon. Immunohistochemical studies have revealed the presence of PKC δ especially in the terminally differentiated cells located at the top of colonic crypts (Kahl-Rainer *et al.* 1996, Verstovsek *et al.* 1998), suggesting that PKC δ is needed for normal colonocyte differentiation. PKC δ has also been implicated in mediating apoptosis in some colon cancer cell lines (Weller *et al.* 1999). In colonic tumours of both human and animal origin, PKC δ has been mostly down regulated, presumably due to prolonged activation of the enzyme at the early phase of the tumourigenic process (Kahl-Rainer *et al.* 1994, Wali *et al.* 1995). Taken together, increased PKC activity in the inulin-fed rats may indicate that these animals are prone to down-regulation of PKC δ.

In line with PKC activity, DAG concentration in the contents of distal colon was the lowest in rats fed wheat bran and elevated in rats fed the oat bran and inulin diets. However, the rye bran-fed rats were an exception since they had the highest faecal DAG level but their PKC activity was as low as in the wheat bran group. Furthermore, though at least partly due to the rye bran group, no significant correlation could be found between DAG and PKC activity, suggesting that luminal DAG is not the major modulator of mucosal PKC activity. Only two previous *in vivo*-studies have attempted to determine the effects of fibre on faecal DAG even though neither analysed mucosal PKC activity. One of these showed that supplementation of wheat bran at 15 g per day for eight weeks reduces faecal DAG concentration in women (Reddy *et al.* 1994), which is in agreement with our results. The other study showed that cellulose relative to pectin at the level 6g/100g in diet enhanced faecal DAG excretion in rats (Pickering *et al.* 1995). The authors concluded that this is probably due to a decrease in the uptake of DAG by epithelial cells and might partly explain the inhibiting effect of cellulose on colon carcinogenesis. The cellulose content as well as other dietary fibre components are largely similar in wheat and rye brans and therefore their opposite effects on luminal DAG in our study are not easily explained. It might be related to a specific effect of either of these brans on gut microbes producing the luminal DAG.

Because the effects of the fibre sources are not fully explained by changes in faecal DAG, there have to be other mechanisms whereby these fibre sources affect PKC. In addition to DAG,

concentrations of the three main SCFAs butyrate, propionate, and acetate, as well as activities of certain faecal bacterial enzymes were analysed in the colonic contents of the same rats used for PKC analysis (M Mutanen, A-M Pajari, S Gråsten, and K Liukkonen, unpublished data). No relation between any of the SCFAs and PKC activity or isozyme levels could be found. This is in agreement with a recent *in vitro* study which showed that PKC does not mediate the differentiating effects of butyrate on colon cancer cells, though its activity augments the effects of butyrate (Rickard *et al.* 1999). In some other cell types, butyrate has greatly stimulated PKC activity and modulated the expression of the PKCs β and ε isozymes (Rivero *et al.* 1998). The bacterial enzymes analysed in our work were β-glucuronidase, β-glucosidase, and urease. Of these, faecal urease activity was significantly but not very strongly ($r = 0.4$, $p < 0.05$) correlated with membrane PKC activity and membrane PKC β2 levels in the distal colon. Even though all the fibre sources tended to increase faecal urease activity compared to the non-fibre diet, the oat bran diet resulted in the highest urease activity. This is of interest because readily fermentable fibres have been shown to promote bacterial growth driven diffusion of blood urea into the caecum (Lupton and Marchant 1989, Younes *et al.* 1996), which may lead to increased levels of luminal ammonia by urease-positive bacteria (Lupton and Marchant 1989). Ammonia, in turn, may damage mucosal cells (Lin and Visek 1991a and 1991b) and result in increased proliferation as a compensatory mechanism for cell loss. Thus, the relation between luminal urease activity and mucosal PKC is probably indirect in nature. Faecal bile acids were not analysed and therefore the possibility that the fibre sources have exerted differential effects on the formation and binding of secondary bile acids can not be excluded. Secondary bile acids are known to be particularly potent activators of PKC β (Pongacrz *et al.* 1995).

The diet induced changes in membrane PKC activity and PKC β2 in the distal colon of rats appeared to predict well the tumour outcome in the intestine of Min mice. Both inulin and oat bran feeding clearly enhanced tumour formation in the intestine of Min mice compared to the non-fibre diet, whereas wheat and especially rye bran resulted in inhibition of tumour formation. Our results are in good agreement with a number of previous experimental studies on the role of fibres in colon carcinogenesis. Wheat bran has protected against tumour development in nearly every experimental model used, including both the rat colon carcinogen model (McIntry *et al.* 1993, Zoran *et al.* 1997) and APC knockout mice (Hioki *et al.* 1997). Rye bran has been studied

once and it was found to be protective in the AOM-model of rat colon carcinogenesis (Davies *et al.* 1999). Readily fermentable fibre sources, such as oat bran, guar gum, and pectin, have not offered protection but in some cases they have promoted colonic tumour formation (Jacobs and Lupton 1986, McIntry *et al.* 1993, Zoran *et al.* 1997). Inulin, a chicory fructan, is highly fermentable but it has been suggested to selectively stimulate the growth of beneficial bacteria in the colon and thus improve the host's health (Gibson and Roberfroid 1995). Inulin has been found to reduce the formation of preneoplastic ACF when added to a basal AIN-76 diet (Reddy *et al.* 1997) or a high-fat corn oil diet (Rowland *et al.* 1998), indicating that inulin inhibits colon carcinogenesis. In respect to inulin the result of the present study contradicts those of previous studies, which could be explained by several reasons. First, the low fat concentration and the presence of cellulose in the AIN-76 diet as well as the high n-6 fatty acid content of the corn oil diet may not optimally reflect a human high-risk diet that is considered to be high in saturated fat and low in fibre. Second, the correlation between the multiplicity of ACF and the tumours that finally develop has not always been straightforward (Hardman *et al.* 1991).

The fibre sources had no significant effects on protein levels of PKC α, β2, δ, and ζ isozymes in Min mice. This is probably due to the fact that the neoplastic process in Min mice at the end of the feeding period was beyond the state in which diet can modulate PKC expression. This is supported by the observation that protein levels of PKC α and δ were under the detection limit in the membrane fraction and they could be measured only from the cytosolic fraction, indicating their full down regulation. A defect in PKC isozyme analysis was that tumour tissue was not separated from the surrounding mucosa, which might have masked the dietary effects. A very recent study by Klein and co-workers (2000) demonstrated reduced protein expression of PKC α, β1, and ζ specific for adenoma tissue in Min mice.

Mice fed the rye bran diet had the lowest and mice fed the inulin and oat bran diets had the highest level of cytosolic β-catenin, suggesting that the immediate mediator of the effects of the fibre sources on tumour formation in Min mice is their ability to modulate β-catenin levels. In Min mice, Apc protein functions poorly due to a mutation in the *Apc* gene, which leads to abnormal accumulation of cytosolic free β-catenin (Mahmoud *et al.* 1997). This β-catenin translocates to the nucleus, where it binds to the Tcf transcription factor and promotes cell

proliferation by enhancing expression of target genes, such as c-*myc* (He *et al.* 1998). The present study is one of the first to demonstrate that β-catenin, the key molecule in this process, can be modulated by dietary means. Exactly how the fibre sources affect β-catenin levels is yet unclear. However, it is of interest that all PKC isozymes analysed were positively correlated with cytosolic β-catenin levels. A number of recent studies have demonstrated that PKC isozymes interact with the APC/β-catenin pathway and that they may have distinct roles in regulating β-catenin level or function. Specifically, PKCs have been shown *in vitro* to mediate inhibition of GSK3β activity (Cook *et al.* 1996), presumably by causing its phosphorylation (Goode *et al.* 1992). In intestinal epithelial cell line, activation of PKC with phorbol 12-myristate 13-acetate triggered the translocation of β-catenin to the nucleus and enhanced β-catenin/Tcf-4 mediated transcription (Baulida *et al.* 1999). The most compelling evidence comes from an *in vivo* study, in which transgenic mice overexpressing PKC β2 had decreased GSK3β activity as well as elevated β-catenin levels (Murray *et al.* 1999).

9. CONCLUSIONS AND FUTURE PERSPECTIVES

Based on the results, it appears that fats do have no major effect on PKC activation or tumour development in the intestinal mucosa of rats and Min mice. However, beef resulted in elevation of steady-state PKC activity in the colonic mucosa of rats as well as enhanced intestinal tumour development in Min mice. The effects of different fibre sources on PKC and tumour formation differed depending on the fibre type in question. The readily fermentable fibre sources inulin and oat bran resulted in an increase in PKC activity and PKC β2 expression in rat colon and enhanced tumour development in the intestine of Min mice, whereas wheat and rye brans resulted in low PKC activity and PKC β2 expression together with suppressed tumour development in Min mice.

Simply an increase in total PKC activity in the colonic mucosa of healthy animals seems to predict surprisingly well the tumour outcome, which could be seen in the animals fed beef and inulin. Accordingly, a decrease in PKC activity in normal colonic mucosa would be expected to lead to inhibition of tumour development, which was at least partly true in respect of rye and wheat brans. Of the PKC isozymes, PKC β2 is perhaps most closely linked to the carcinogenic process in the colon, since an increase in PKC β2 expression was as good predictor of tumour outcome as PKC activity. One reason for this may be that PKC β2 seems to be an important mediator of colon cell proliferation. Disturbances in proliferation may result in a hyperproliferating colonic epithelium, which is considered to be the very first stage in the transforming process of normal epithelium to colon cancer. Also, changes in PKC ζ and λ expression may be of importance, as suggested by the results concerning beef feeding. The specific functions of atypical PKCs in the colon are far less clear than those of conventional and novel PKCs. Presumably atypical PKCs regulate maturation and growth of colonocytes. The changes in PKC in the distal colonic mucosa appear to be the most important determinants of tumour outcome. This is not surprising in view of the fact that colon tumours in high-incidence western countries are more common in the distal colon than in other sites (Bufill 1990).

It seems that PKC in the late phase of the neoplastic process no longer predict the tumour outcome as could be seen in Min mice at the end of the feeding period. This result is, however, tentative since there were limitations in PKC analysis in the Min mice study. It is noteworthy that

PKC isozyme levels did correlate with cytosolic β-catenin level. This observation supports the results from previous *in vitro* studies that have linked PKC to the APC pathway, one of the major cellular pathways involved in colon carcinogenesis.

In future studies, it is important to determine the changes in cellular events occurring at different time points in the tumourigenic process. Specifically, it would be interesting to see how PKC activity and certain isozymes change during the tumourigenic process and whether these changes are linked to alterations in the APC-pathway, especially β-catenin levels. Furthermore, the result of rye bran feeding being able to reduce β-catenin levels in Min mice suggests that diet might be a powerful modulator of the molecular events leading to colon cancer. The exact effects of dietary factors on these events are worthy of further studies.

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