Dietary Fat and Fiber Modulate the Effect of Carcinogen on Colonic Protein Kinase C λ Expression in Rats[1,2,3]

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ABSTRACT To elucidate the mechanisms by which dietary factors influence the risk of colon cancer, we investigated the effect of select dietary fats and fibers on atypical protein kinase C (PKC) λ expression. Azoxymethane- and saline (control)-injected rats were fed diets containing either corn oil or fish oil (15 g/100 g) and either cellulose or pectin (6 g/100 g) and killed at two time points (15 and 37 wk) in a 2 × 2 × 2 × 2 factorial design. Colonic PKC λ protein and mRNA levels were determined using immunoblotting and relative competitive polymerase chain reaction, respectively. Azoxymethane suppressed cytosolic PKC λ protein levels compared with the saline controls at both time points, and this suppression was partially blocked by fish oil feeding at 15 wk and pectin at 37 wk. Also, at 15 wk, azoxymethane-injected rats fed corn oil had higher levels of membrane PKC λ relative to the other treatment groups. Overall, expression of PKC λ mRNA was not correlated with differences in the respective isozyme protein levels. Therefore, the chemopreventive effects of dietary fish oil and pectin are associated with the blockage of azoxymethane-induced alterations in colonic PKC λ protein expression. J. Nutr. 127: 1938–1943, 1997.

KEY WORDS: • *protein kinase C λ* • *colon* • *azoxymethane* • *fish oil* • *pectin* • *rats*

The mechanisms by which dietary fish oil, containing (n-3) polyunsaturated fatty acids (PUFA),[5] and select dietary fibers reduce the incidence of colon cancer are still unclear (Caygill et al. 1996, Potter et al. 1993). We previously demonstrated that dietary (n-3) PUFA compared with (n-6) PUFA can alter the balance between colonic cell division, maturation and programmed cell death (Chang et al. 1997, Chapkin et al. 1993, Lee et al. 1993a and 1993b). In addition, the effect of dietary fiber on colonic cell proliferation is highly dependent on the source of fat in the diet (Chang et al. 1997, Lee et al. 1993a and 1993b, Pickering et al. 1995). We recently reported, as part of a larger parent study, that fiber effects on colon tumorigenesis are also highly dependent on the fat source in the diet (Chang et al. 1997). Previously, we demonstrated that pectin supplementation with a corn oil diet is promotive of colon cancer compared with a fiber-free diet (Jacobs and Lupton 1986). In contrast, when pectin is combined with fish oil, there is a protective effect with respect to the number of animals developing adenocarcinomas (Chang et al. 1997).

Because epigenetic alterations in protein kinase C (PKC) are associated with colon carcinogenesis in both humans and experimental animals (Baum et al. 1990, Blobe et al. 1994, Craven and DeRubertis 1987, Sakanoue et al. 1991, Wali et al. 1995), we have postulated that the ability of select combinations of dietary fats and fibers to reduce colon carcinogenesis may be mediated by changes in PKC-related signal transduction during the initial stages of tumorigenesis.

The PKC isozymes compose a family of at least 11 different serine/threonine kinases that have been implicated in the modulation of colonic cell proliferation, differentiation and programmed cell death (Blobe et al. 1994, Chapkin et al. 1993, Jiang et al. 1996b, Jiang et al. 1997, Wali et al. 1995). The PKC family can be classified into three major groups: *1)* classical (cPKC-α, -βI, -βII, and -γ), requiring phosphatidylserine, calcium and diacylglycerol for activation; *2)* novel (nPKC-δ, -ϵ, -θ and -μ), which do not require calcium and exhibit enzyme activities in the presence of phosphatidylserine and diacylglycerol; and *3)* atypical (aPKC-ζ and λ/ι), which require neither calcium nor conventional sources of phospholipid for activation and are phorbol ester–insensitive. Recently, the presence of a novel signaling pathway from receptor tyrosine kinases (e.g., epidermal growth factor receptor, EGFR) through phosphatidylinositol 3-kinase to an atypical PKC (PKC λ) has been demonstrated (Akimoto et al. 1996). This is significant because changes in the level of epidermal growth factor–related gene expression in specific colonic epithelial populations may be important in the pathogenesis of colon cancer (Gross et al. 1991, Saeki et al. 1992). Recently, we reported that dietary fish oil blocks the carcinogen-induced decrease in the steady-state protein levels of colonic mucosal PKC δ and a mixture of immunoreactive λ/ζ, which may in part explain why this fat source protects against colon cancer development

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[5] Abbreviations used: AOM, azoxymethane; EGFR, epidermal growth factor receptor; PUFA, polyunsaturated fatty acid; PKC, protein kinase C; RC-PCR, relative competitive polymerase chain reaction.

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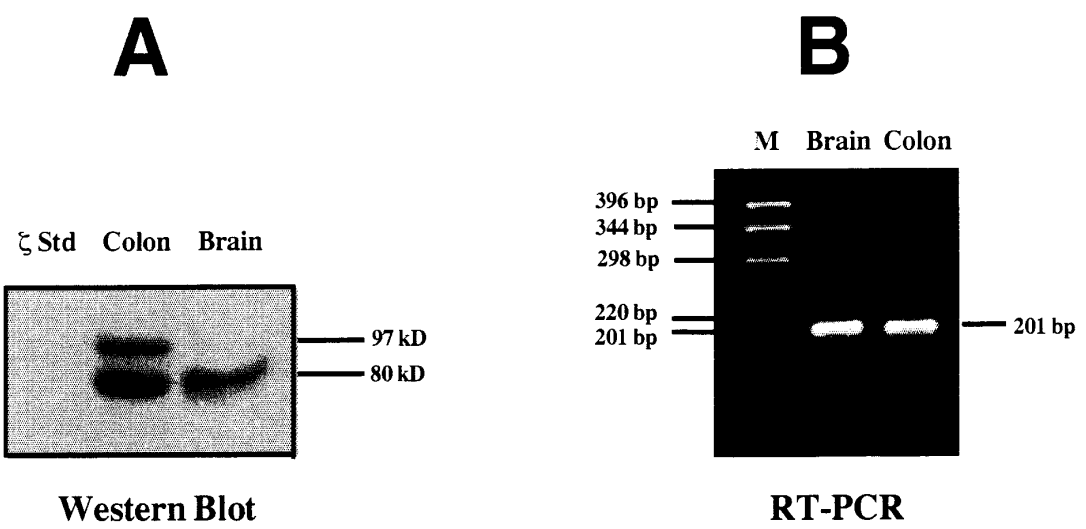

FIGURE 1 *A:* Rat colonic mucosal protein kinase C (PKC) λ expression as determined by immunoblot analysis. From left to right: lane 1, PKC ζ recombinant standard (1 μg protein); lane 2, colonic mucosal homogenate (5 μg protein); lane 3, extract from rat brain (5 μg protein). *B:* Relative competitive polymerase chain reaction (RT-PCR) of PKC λ mRNA in representative samples. From left to right: lane 1, 1-kb molecular markers (m); lane 2, rat brain; lane 3, colonic mucosa.

(Jiang et al. 1997). However, to date, the specific detection of PKC λ in rat colon, its nucleotide identity with other atypical PKC isozymes, and its modulation by diet and carcinogen have not been examined.

In this report, we determined the effects of specific dietary fats and fibers on colonic PKC λ expression in the early and late stages of rat colorectal cancer development.

MATERIALS AND METHODS

Animals and diet administration. The animal use protocol was approved by the University Animal Care Committee of Texas A&M University and conformed to NIH guidelines. One hundred and sixty male weanling Sprague-Dawley rats (Harlan Sprague Dawley) were stratified by their body weight and assigned to one of four different experimental diets supplemented with either corn oil or a corn oil-fish oil mixture (at 15 g/100 g) and either cellulose or pectin (at 6 g/100 g) in a 2 × 2 × 2 × 2 factorial design as previously described (Maciorowski et al. 1997). The rats were fed the diets for the duration of the experiment (44 wk). Rats received fresh diet daily. Vacuum-deodorized menhaden fish oil (National Institutes of Health) contained 1 g/kg α- and γ-tocopherol and 0.25 g/kg tertiary butylhydroquinone (TBHQ) as added antioxidants. Food-grade corn oil, which contains some α- and γ-tocopherol, was supplemented further with tocopherols and TBHQ to provide amounts equal to that in fish oil.

Carcinogen administration and tissue procurement. Following 1 wk of acclimatization, 20 rats within each diet group were injected with either azoxymethane (AOM) or saline (control) as previously described (Chang et al. 1997, Jiang et al. 1996b, Pickering et al. 1995). All animals received the experimental diets until the termination of the experiment. After rats were killed by CO_2 asphyxiation, the entire colon was removed, rinsed with PBS, opened longitudinally, examined visually for tumor incidence and divided into two equal pieces (designated proximal and distal). Following removal of suspected tumors for histological evaluation, the remaining proximal and distal sections were scraped and the mucosa used for determination of the steady-state levels of PKC λ mRNA and protein.

Protein extraction and immunoblotting. Homogenized samples from scraped colonic mucosa were ultracentrifuged at 100,000 × *g* for 30 min, and the supernatant was taken as the soluble fraction and frozen in aliquots at −80°C as previously described (Davidson et al. 1994). The pellet was further extracted with buffer supplemented with 1% Triton X-100 (Jiang et al. 1996b). Rat brain homogenates were used as a positive control. Colonic mucosa and brain homogenates were treated with SDS sample buffer and subjected to polyacrylamide gel electrophoresis in 4–12% pre-cast minigels (Novel Experimental Technology, San Diego, CA) and electroblotted per the method of Davidson et al. (1994). Following transfer, the membrane was processed using primary mouse monoclonal antibody PKC λ (Transduction Laboratories, Lexington, KY, 1:2000) in PBS containing 5% nonfat dry milk. An 18.3-kDa protein fragment corresponding to amino acids 397–558 of mouse PKC λ was used as an immunogen. This antibody does not cross-react with atypical PKC ζ (**Fig. 1**A). Dilution of primary antibody was titrated for PKC λ, and the membrane was incubated for 2 h at room temperature with rocking. Affinity purified peroxidase-labeled secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was incubated for 1 h at room temperature, followed by ECL Western blotting detection system (Amersham, Arlington Heights, IL) to visualize the PKC λ band. A range of protein concentrations for PKC λ was loaded onto representative gels to ensure that the response was quantitative. Blots were scanned and quantified using Bio Image Intelligent Quantifier™ software (Bio Image, Ann Arbor, MI) to measure band intensity. All results were normalized to a constant amount of protein from pooled rat brain.

Total RNA extraction and PKC isozyme quantification. Total RNA from colonic mucosa was extracted using Totally RNA™ as per manufacturer's instructions (Ambion, Austin, TX). The relative mRNA levels of PKC isozymes were quantified using rapid competitive polymerase chain reaction (RC-PCR) as previously described (Jiang et al. 1996a and 1997). Primer pairs for construction of internal standards and amplification of PKC λ mRNA were as follows: PKC λ competitor (174 bp) forward, 5′-GCTTATGTTTGAGATGATGGCGGGCTCTGACAATCCTG-3′; reverse, 5′-TGACAACCCAATCGTTCCTTTG-3′; PKC λ (201 bp) forward, 5′-GCTTATGTTTGAGATGATGGCGG-3′; reverse, 5′-TGACAACCCAATCGTTCCTTTG-3′. The fidelity of all PCR amplifications were confirmed by DNA sequencing (Davidson et al. 1994).

Statistical analysis. Data were analyzed to determine the effect of dietary fat, fiber and carcinogen and the fat × fiber, fat × carcinogen, fiber × carcinogen, and fat × fiber × carcinogen interactions using three-way ANOVA. The proximal and distal mucosa were separately assayed, and the data were combined. When the *P* values for the interactions were <0.05, means of all diet groups were then separated using Duncan's multiple range test (SAS 1985). When the *P* values were <0.05 for the effects of fat, fiber or carcinogen but not for the interactions, total means of fat or fiber or carcinogen-treated groups were separated using Duncan's multiple range test.

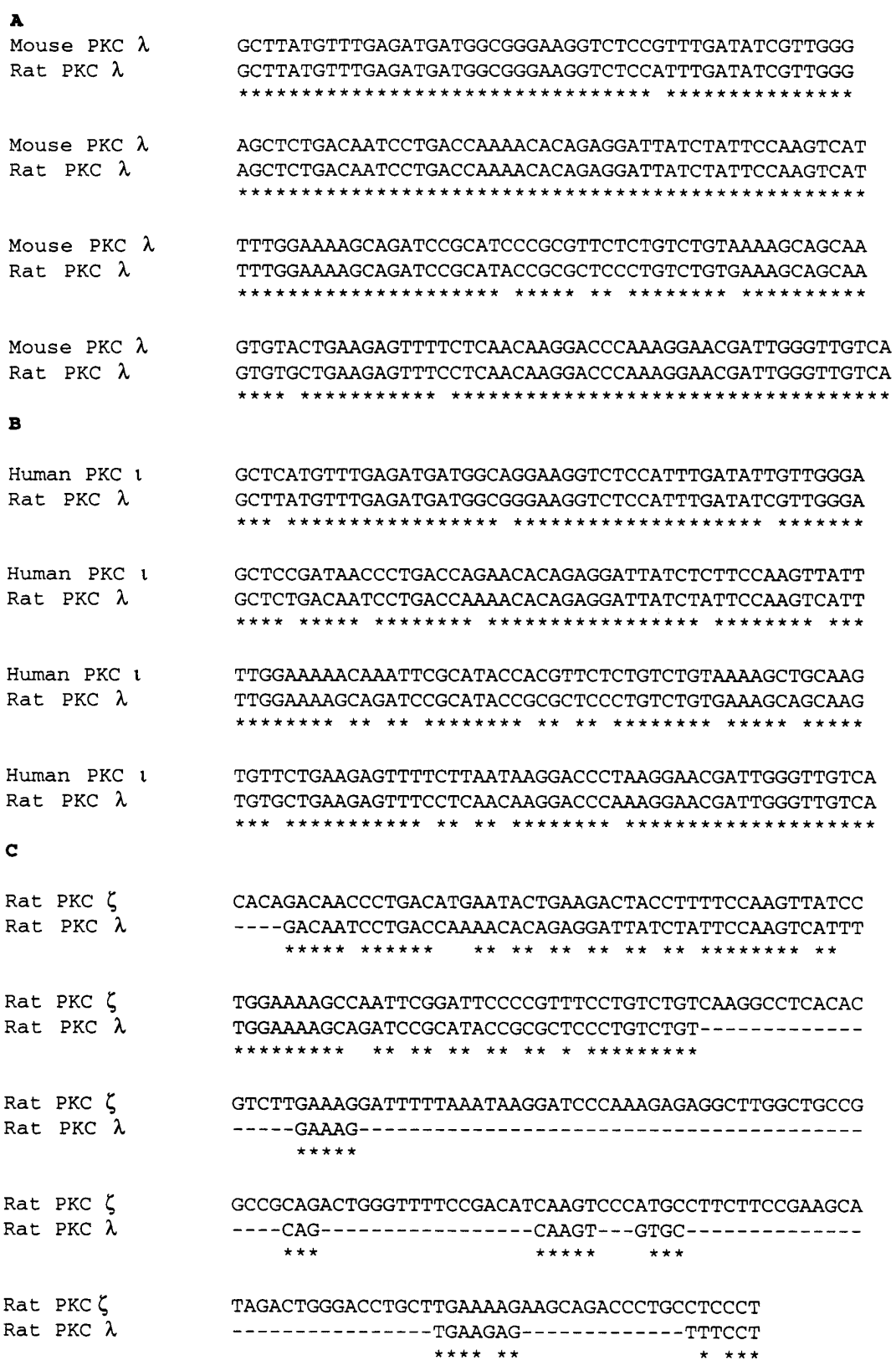

FIGURE 2 Partial sequence alignment of protein kinase C (PKC) λ C3 and D4 regions with other atypical PKC isozymes. *A:* Alignment of mouse PKC λ with rat PKC λ. The partial nucleotide identity between rat PKC λ and mouse PKC λ was 96.5%. *B:* Human PKC ι alignment with rat PKC λ. The partial nucleotide identity between rat PKC λ and human PKC ι was 89.1%. *C:* Rat PKC ζ alignment with rat PKC λ. The partial nucleotide identity of rat PKC λ with rat PKC ζ was 60.2%.

TABLE 1

Effect of carcinogen and dietary fat and fiber on protein kinase C (PKC) λ protein expression in rat colonic mucosa 15 or 37 wk after azoxymethone (AOM) or saline (control) injection[1,2,3]

	Cytosol		Membrane	
	15 weeks[4]	37 weeks	15 weeks	37 weeks
	relative units			
Carcinogen				
AOM	0.860 ± 0.111$^{b\#}$	1.834 ± 0.303^{b*}	0.323 ± 0.022^{b+}	0.278 ± 0.049^{b+}
Saline	1.305 ± 0.111$^{a\#}$	2.544 ± 0.030^{a*}	0.223 ± 0.022^{a+}	0.596 ± 0.049^{a+}
Fat				
Corn oil	0.815 ± 0.111$^{b\#}$	2.450 ± 0.303^{a*}	0.340 ± 0.022^{b+}	0.478 ± 0.049^{a+}
Fish oil	1.350 ± 0.111^{a*}	1.928 ± 0.303^{a*}	0.205 ± 0.022^{a+}	0.396 ± 0.049^{a+}
Fiber				
Cellulose	1.077 ± 0.111^{a*}	1.357 ± 0.303^{b*}	0.275 ± 0.022^{a+}	0.412 ± 0.049^{a+}
Pectin	1.088 ± 0.111$^{a\#}$	3.021 ± 0.303^{a*}	0.271 ± 0.022^{a+}	0.462 ± 0.049^{a+}

[1] Colonic mucosa PKC λ protein content is expressed relative to rat brain (control). Dietary treatments were initiated 1 wk before carcinogen (AOM) injection.
[2] Means ± SEM (n = 32–40) in a main effect column not sharing a common superscript letter are significantly different ($P < 0.05$).
[3] Means ± SEM (n = 16–20) in a row not sharing a common superscript symbol are significantly different ($P < 0.05$).

RESULTS

At 15 wk after injection, no evidence of severe atypia, carcinoma in situ, or microscopic adenocarcinoma was seen by light microscopic examination. However, alterations in colonic cell proliferation, differentiation and apoptosis were noted in AOM-injected compared with saline-injected rats (Chang et al. 1997). At 37 wk after injection, colonic adenomas and carcinomas were detected in carcinogen (AOM)-injected rats (Chang et al. 1997, Jiang et al. 1996b). For the rats injected with AOM, there was a significant main effect of fat ($P < 0.05$) on adenocarcinoma incidence, with fish oil resulting in a tumor incidence of 56.1% compared with 69.9% for corn oil. There was no main effect of fiber on tumor incidence ($P > 0.05$), although when all four treatments were separated, rats fed corn oil and cellulose had a higher ($P < 0.05$) adenocarcinoma incidence (75.6%) than rats fed the combination of fish oil and pectin (51.5%) (Chang et al. 1997).

Detection of rat colonic PKC λ. The amino acid sequence of mouse PKC λ has greatest homology to PKC ζ, with 72% amino acid identity (Selbie et al. 1993). The sequence identities between the kinase (C3) region of PKC λ and those of other members were 86% for PKC ζ and 44% (θ) to 55% (ϵ) for other family members (Selbie et al. 1993). Therefore, the specificity of PKC λ antibody was confirmed by determining immunoreactivity toward both recombinant PKC ζ standard and rat colon and brain extracts. As shown in Figure 1A, PKC

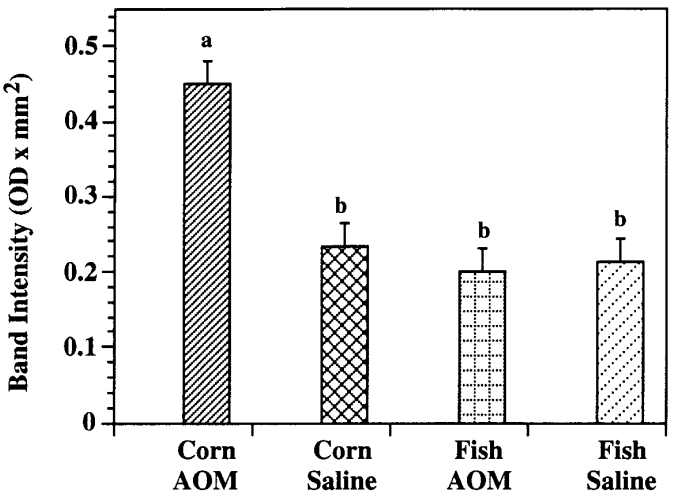

FIGURE 3 Interactive effect of dietary fat and carcinogen on protein kinase C (PKC) λ membrane expression in colon of rats at 15 wk after injection as determined by immunoblotting. Corn AOM: rats injected with azoxymethane (AOM) and fed the corn oil diet. Corn Saline: rats injected with saline and fed the corn oil diet. Fish AOM: rats injected with AOM and fed the fish oil diet. Fish Saline: rats injected with saline and fed the fish oil diet. Values are means ± SEM, n = 16–20 rats/group. Bars with different letters are significantly different ($P < 0.05$).

TABLE 2

Effect of carcinogen and dietary fat and fiber on protein kinase C (PKC) λ mRNA expression in rat colonic mucosa 15 or 37 wk after azoxymethane (AOM) or saline (control) injection[1,2]

	15 weeks	37 weeks
	relative units	
Carcinogen		
AOM	1.574 ± 0.272^{a}	1.326 ± 0.176^{a}
Saline	1.868 ± 0.267^{a}	1.214 ± 0.195^{a}
Fat		
Corn Oil	2.263 ± 0.258^{b}	1.616 ± 0.190^{b}
Fish Oil	1.180 ± 0.281^{a}	0.923 ± 0.181^{a}
Fiber		
Cellulose	1.614 ± 0.271^{a}	1.284 ± 0.185^{a}
Pectin	1.829 ± 0.269^{a}	1.256 ± 0.187^{a}

[1] Colonic PKC λ mRNA expression is expressed relative to rat brain (control). Dietary treatments were initiated 1 wk before carcinogen (AOM) injection.
[2] Means ± SEM (n = 33–39) in a main effect column not sharing a common superscript letter are significantly different ($P < 0.05$).

λ was detected in rat colon protein extracts. Recombinant PKC ζ standard served as a negative control. To detect PKC λ mRNA in rat colon, primers were designed using sequence from mouse PKC λ (accession number D28577). Polymerase chain reaction amplification generated a 201-bp product, as shown in Figure 1*B*. The fidelity of amplification was confirmed by DNA sequencing (described below).

Comparison of rat PKC λ to mouse PKC λ, human PKC ι and rat PKC ζ isozymes. The PCR products corresponding to the C3 and D4 regions of PKC λ were amplified. Partial sequence analysis of rat PKC λ was performed and the aligned sequences of the atypical PKC isozymes are shown in **Figure 2.** The partial nucleotide identity between rat PKC λ and mouse PKC λ was 96.5% (Fig. 2*A*). The partial nucleotide identity between rat PKC λ and human PKC ι was 89.1% (Fig. 2*B*), and the homology of rat PKC λ with rat PKC ζ was 60.2% (Fig. 2*C*).

Effect of carcinogen, fat and fiber on PKC λ expression in rat colon. The PKC λ protein levels were quantified in rat colonic mucosa homogenates from both membrane and cytosolic fractions by immunoblot analysis. Rat colon and brain (positive control) expressed PKC λ, detected as an ~80-kDa band. The steady-state level of PKC λ protein in both membrane and cytosolic fractions was affected by carcinogen (AOM) treatment at both 15 and 37 wk after injection (**Table 1**). Rats injected with AOM had significantly ($P < 0.05$) lower PKC λ expression relative to saline-injected rats, except for the membrane fraction at 15 wk. In addition, a fat × AOM interaction was detected, with rats injected with AOM and fed corn oil having significantly ($P < 0.05$) higher PKC λ membrane levels relative to the other treatment groups at 15 wk after injection (**Fig. 3**). Dietary fat and fiber composition also influenced PKC λ localization (Table 1), with fish oil consumption significantly ($P < 0.05$) elevating PKC λ protein expression in the cytosolic fraction as compared with corn oil at 15 wk. In contrast, corn oil consumption significantly ($P < 0.05$) increased PKC λ membrane association at 15 wk. At 37 wk, pectin-fed rats had a significantly ($P < 0.05$) higher cytosolic PKC λ expression as compared with those fed cellulose.

The relative content of PKC λ mRNA in rat colon was measured using rapid competitive PCR. In general, PKC λ mRNA expression (**Table 2**) was not correlated with differences in protein expression (Table 1). Rats fed corn oil diets had significantly higher ($P < 0.05$) steady-state levels of PKC λ mRNA as compared with those fed fish oil diets at both time points.

Translocation of PKC λ. Previous studies have shown that many PKC isozymes translocate to the membrane upon activation (Kazanietz and Blumberg 1996). In this study, ratios were calculated for all treatment groups; however, only the significantly different ratios are shown. The AOM-injected rats had a significantly ($P < 0.05$) higher PKC λ membrane/cytosol ratio (1.19 ± 0.24) than did the saline-injected rats (0.36 ± 0.24) at the intermediate time point (15 wk). In contrast, AOM injection decreased ($P < 0.05$) the membrane/cytosol ratio as compared with saline administration (0.22 ± 0.06 vs. 0.43 ± 0.06) at the final time point (37 wk).

DISCUSSION

Experimental findings indicate that PKC may play a central role in colon carcinogenesis (Baum et al. 1990, Blobe et al. 1994, Craven and DeRubertis 1987, Jiang et al. 1997, Sakanoue et al. 1991, Wali et al. 1995). We have recently demonstrated that dietary (n-3) PUFA block the carcinogen-induced decrease in the steady-state levels of select colonic PKC isozymes (Jiang et al. 1997). We extend these observations by demonstrating that diet and carcinogen alter rat colonic mucosa PKC λ expression and intracellular localization. Protein kinase C λ is a recently identified atypical member of the PKC family of isozymes and is activated through a signaling network distinct from the classical pathways involving diacylglycerol (Akimoto et al. 1996, Selbie et al. 1993). Recent evidence indicates that PKC λ is involved in signaling from the EGFR through phosphatidylinositol 3-kinase to the nucleus (Akimoto et al. 1996). This is noteworthy, because alterations in EGFR-dependent intracellular signal transduction have been implicated in the development of colon cancer (Gross et al. 1991, Saeki et al. 1992).

We have demonstrated that dietary fat and fiber uniquely influence intracellular second messengers at different stages of colon carcinogenesis (Jiang et al. 1996b and 1997). In the present study, dietary fish oil and pectin modulated PKC λ expression at different stages of colon cancer. Specifically, prior to the development of overt neoplasia (15 wk), AOM treatment significantly elevated PKC λ membrane localization. This effect was blocked by fish oil feeding. Because in conventional in vitro assays the activation of many PKC isozymes in cells is commonly associated with a redistribution (translocation) of cytosolic enzymes to membranes (Kazanietz and Blumberg 1996), these data suggest that carcinogen exposure increases colonic PKC λ activation. It is also becoming clear that long-term changes in PKC isozyme levels and localization provide a mechanism for long-term regulation (Kazanietz and Blumberg 1996). Interestingly, in the later stages of malignant transformation (37 wk), when tumors are apparent (Chang et al. 1997, Jiang et al. 1996b), carcinogen administration reduced both cytosolic and membrane PKC λ expression. In contrast, pectin consumption increased cytosolic PKC λ levels relative to cellulose consumption. The late-stage carcinogen-mediated decrease in immunoreactive PKC λ is consistent with lower PKC activity in colon carcinomas (Baum et al. 1990, Sakanoue et al. 1991, Wali et al. 1995). It is possible that the long-term stimulation of PKC λ by the presence of carcinogen may initially activate PKC but subsequently cause long-term down-regulation of the isozyme.

The lack of a correlation between the steady-state levels of colonic PKC λ mRNA and protein amount indicates the likelihood of regulation at the post-transcriptional level, possibly via proteolytic cleavage (Jiang et al. 1995 and 1997, Young et al. 1987). Although dietary fish oil and pectin can partially block the effects of carcinogen, the biological significance of alterations in PKC λ subcellular compartmentalization remains to be determined. However, it bears emphasis that among dietary factors, there is cogent experimental, clinical and epidemiological evidence indicating a protective effect of (n-3) PUFA and fiber on colon cancer incidence (Anti et al. 1994, Caygill et al. 1996, Chang et al. 1997, Jiang et al. 1996b, Potter et al. 1993, Reddy 1992). In addition, we have recently demonstrated that dietary fish oil and pectin can synergize to reduce several critical colonic intracellular signaling molecules (e.g., phospholipase C-γ1 expression and diacylglycerol mass) (Jiang et al. 1996b), which were correlated with a consistent elevation in cell differentiation and reduced tumor formation (Chang et al. 1997, Jiang et al. 1996b). In conclusion, the chemopreventive effects of the combination of dietary fish oil and pectin are associated with alterations in colonic PKC λ expression, a signal-dependent kinase that is activated upon stimulation by growth factors.

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