Selective preservation of protein kinase C-ζ in the chemoprevention of azoxymethane-induced colonic tumors by piroxicam

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Abstract While nonsteroidal anti-inflammatory drugs have been shown to exert preventive effects against the development of colonic tumors in humans and in chemically-induced tumors in animal models, the mechanism(s) involved in this phenomenon is unclear. We have recently demonstrated that one such agent, piroxicam, when supplemented (75 ppm) in the diets of rats administered azoxymethane, reduced the incidence of rats bearing tumors. To date, the effects of piroxicam on protein kinase C, a family of serine/threonine kinases which may be intimately involved in the colonic malignant transformation process, have not been examined. It was, therefore, of interest to determine whether piroxicam altered the expression of one or more isoforms of this kinase in these tumors. The present studies demonstrate that dietary piroxicam selectively preserved the expression of protein kinase C-ζ in azoxymethane-induced tumors; suggesting that this is at least one mechanism involved in this agent's chemopreventive actions in this organ.

Key words: Signal transduction; PKC; Chemical carcinogenesis; Large bowel; Nonsteroidal anti-inflammatory agent

1. Introduction

Colorectal cancer is a major cause of morbidity in the United States [1]. Despite advances in the fields of surgery, radiotherapy and chemotherapy, cure rates for this disease(s), unfortunately, have not significantly improved during the past several decades [1]. Based on these considerations, several potential anticarcinogens have been studied in an effort to prevent colorectal carcinoma. Nonsteroidal anti-inflammatory drugs (NSAIDs)[1], including piroxicam, aspirin, sulindac and indomethacin, have shown promise for this purpose. A number of epidemiological studies in humans [2–4], interventional investigations in patients with familial polyposis [5–6], as well as animal studies using various models of colonic carcinogenesis [7–10], have suggested that NSAIDs reduce the risk of developing colorectal cancer and/or its associated mortality. The exact

mechanism(s) involved in these chemopreventive actions of NSAIDs, however, remain unclear and controversial [11].

In this regard, there are several lines of evidence which indicate that alterations in the activity and/or the expression of one or more isoforms of protein kinase C (PKC) may be intimately involved in the colonic malignant transformations process(es) in man and experimental animals [12–17]. To date, however, the effects of NSAIDs on PKC have not been examined. Recently, in agreement with prior studies [8], our laboratory [10] has demonstrated that dietary supplementation with piroxicam (75 ppm) significantly reduced the incidence of rats bearing tumors as well as tumor size in the azoxymethane (AOM)-model of experimental colonic tumorigenesis. In the present experiments, therefore, it was of interest to examine and compare the protein expression of several PKC isoforms in these colonic tumors from animals treated with AOM alone, or treated with AOM and supplemented with piroxicam in their diet.

2. Materials and methods

As recently described in detail [10], weanling male albino Fisher (F344) rats, initially weighing 90–130 g, were randomly assigned into groups fed an AIN-76 standard diet (Bio-serve Co., Frenchtown, NJ) with or without supplemental piroxicam (75 ppm, Sigma Chemical Co., St. Louis, MO). Two weeks after being placed on their diets, one-half the rats in each group were s.c. injected with AOM (15 mg/kg body wt/week) or vehicle (saline) for 2 weeks and then maintained on their respective diets for an additional 28 weeks. At that time, the animals were sacrificed, macroscopic tumors excised and harvested individually, and sections of each tumor were fixed in formalin and subjected to histological examination [10]. Homogenates of the remaining portion of each tumor were prepared by sonication in SDS containing buffer and proteins assayed using a previously described method [18]. Proteins (~20 μg) were resolved by SDS-PAGE with a 10% separating gel, transferred to PVDF membranes (Millipore Co., Bedford, MA) and probed with isoform specific antibodies, i.e. monoclonal anti-PKC-a antibodies (Upstate Biotechnology, Lake Placid, NY) and polyclonal antipeptide antibodies to PKC-δ, PKC-ε and PKC-ζ (Gibco BRL, Gaithersburg, MA) as previously described [19]. The major immunoreactive band to PKC-ζ antisera was a protein with M_r 78,000. Inconsistently, and only with prolonged exposure of the xerograms, we also observed a second band with M_r 80,000 that comigrated with PKC-α as previously described [20]. PKC-ζ was identified as the component with M_r 78,000 which we had previously shown did not redistribute in response to acute treatment of colonocytes with TPA [21]. Furthermore, the immunoreactivity of this band with PKC-ζ antisera was abolished by preincubation of the antisera with the ζ-specific immunizing peptide. Quantitative PKC-ζ protein expression reflects the 78,000 Da component only. Secondary antibodies were detected with an enhanced chemiluminescence system (Amersham Co., Arlington Heights, IL), and quantitated by scanning densitometry as previously described [19]. Data was expressed as means ± S.D. and analyzed for statistical significance by the Student's *t*-test for unpaired samples [22].

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3. Results

3.1. The effects of AOM and piroxicam on colonic expression of PKC isoforms

In agreement with prior studies from our laboratory [21], normal rat colonocytes possessed PKC-α, PKC-δ, PKC-ε and PKC-ζ. In vehicle-treated animals, dietary supplementation with piroxicam did not significantly alter the expression of any of the isoforms in these cells (data not shown). As shown in Fig. 1, however, the expression of PKC-α and PKC-δ were decreased, i.e. 'down-regulated', while PKC-ε was unchanged, in
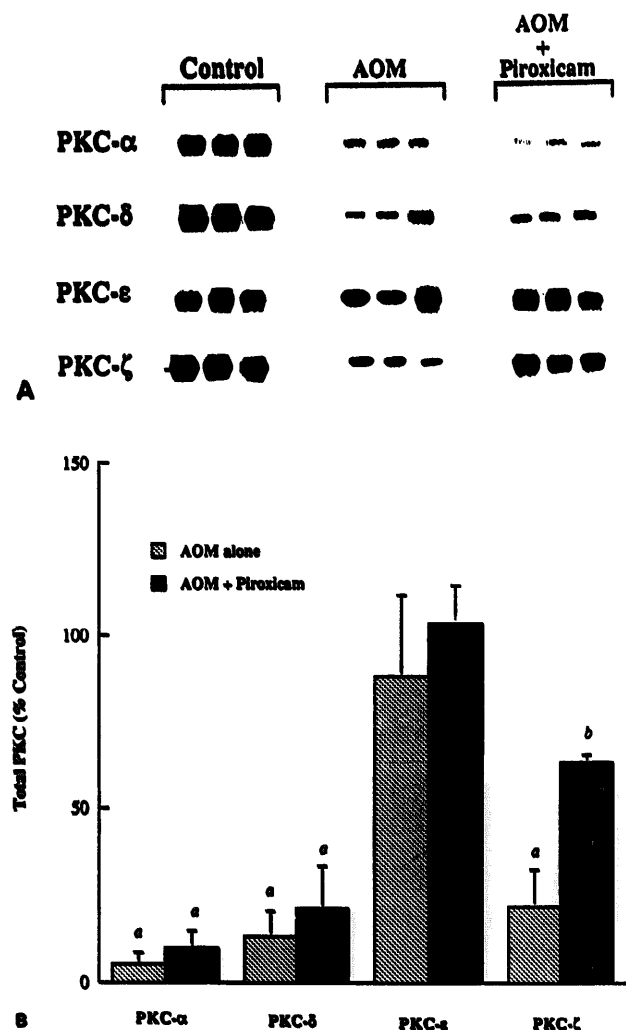

Fig. 1. Effects of piroxicam supplementation on PKC isoform expression in AOM-induced tumors. (A) Representative immunoblots of colonic homogenates (20 μg) from three separate control rats, or AOM-induced tumors, probed with specific antibodies to the indicated PKC isoforms. (B) Quantitative densitometry of PKC isoform expression in AOM-induced tumors from animals on the AIN diet alone, or the piroxicam-supplemented diet. Data (mean ± S.D.) is expressed as% of control with analysis of 5–7 tumors per group. a, P < 0.05, compared with appropriate controls (colonocytes from vehicle-treated animals on either an AIN diet alone, or one supplemented with piroxicam). (b) P < 0.05, compared with PKC-ζ expression in AOM-induced tumors from rats on a diet with no piroxicam supplementation. Note, there was no significant difference in PKC-ζ expression between control values and AOM-induced tumors from the piroxicam supplemented group (P = 0.66, unpaired Student's t-test).

AOM-induced colonic tumors harvested from animals regardless of whether their diets were or were not supplemented with piroxicam. As can also be seen in this figure, while the expression of PKC-ζ (M_r 78,000 component) was also found to be down-regulated in tumors of animals fed the control diet, the expression of this isoform was found to be preserved in AOM-induced tumors of animals fed the piroxicam supplemented dietary regimen.

As noted previously [10], both adenomas and carcinomas were present in the AOM-treated groups fed an unsupplemented or piroxicam supplemented diet. In the present studies, however, there where no significant differences in the total expression of any of the PKC isoforms in the carcinomas (2 analyzed in each group) compared to the adenomas (3–5 analyzed in each group) (data not shown).

4. Discussion

The mechanism(s) by which NSAIDs inhibit the development of colonic tumors in animal models of chemically-induced carcinogenesis, as well as perhaps in man, are presently controversial and currently the subject of intense investigation. Potential mechanisms, to date, which have been postulated to explain the effects of these agents' chemopreventive effects in this organ include: (i) reduction in prostaglandin synthesis via inhibition of cyclooxygenase(s) activity [8,11]; (ii) inhibition of cellular proliferation [11]; (iii) alterations in immunomodulatory cellular events [11,23]; and (iv) changes in the regulation of signal transduction events [11]. In this regard, evidence has accumulated for [8,11,24,25] and against [7,26] the possibility that these agents prevent colon cancer by inhibiting prostaglandin synthesis. Recent preliminary studies in cultured cells [27], as well as by our laboratory in the AOM model of experimental colonic carcinogenesis [28], argue against effects of these agents on cellular proliferation being involved in this phenomenon. Our laboratory has, however, recently demonstrated that dietary piroxicam supplementation (75 ppm) can upregulate the expression of MHC class I and II antigens in premalignant colonocytes in the aforementioned experimental cancer model, suggesting that piroxicam-induced immunomodulatory events may, at least in part, explain its chemopreventive actions in the rat colon [23].

The results of the present studies indicate that dietary piroxicam supplementation may have an additional chemoprotective mechanism of action, i.e. this dietary regimen was found to selectively preserve the expression of PKC-ζ in AOM-induced tumors compared to its loss in tumors from AOM-treated animals maintained on a non-supplemented diet. In this regard, it bears emphasis that in preliminary studies [29] we have also noted a similar effect of ursodeoxycholic acid, another structurally unrelated chemopreventive agent [10], on the preservation of PKC-ζ expression in tumors in this model. It is, therefore, possible that this finding may have more general import with respect to chemoprevention of colonic carcinogenesis. Further studies, particularly in premalignant colonocytes of animals treated with AOM alone, or with dietary piroxicam, will therefore be of interest.

PKC-ζ is a member of the Ca^{2+}-insensitive group of PKC isoforms [30]. Moreover, it is also phorbol ester-insensitive in many cell types [30]. The present findings with respect to this PKC isoform are of interest since there is now accumulating

evidence that PKC-ζ may play a key role in cell growth, differentiation and possibly malignant transformation. In this regard, PKC-ζ can be activated by phosphoinositol 3,4,5-trisphosphate, indicating that this isoform is involved in signalling cascades of growth factors and oncogenes [31]. PKC-ζ has also recently been implicated in the inhibition of intestinal cellular growth and the induction of differentiation [32], as well as shown to be essential in the cellular maturation of *Xenopus* oocytes [33]. It bears emphasis that in these studies piroxicam supplementation was associated with preservation of PKC-ζ, a potential growth-limiting isoform, and a decrease in tumor size as well as a decrease in incidence of rats bearing tumors [10]. This suggests that piroxicam, perhaps through PKC-ζ, may play important roles both to inhibit tumor initiation (decreased incidence of tumor-bearing rats) and promotion (decreased tumor size). In addition, our laboratory has recently shown that PKC-ζ was the only PKC isoform constitutively expressed in the nuclei of CaCo-2 cells, a cell line widely used to investigate intestinal cell growth and differentiation, suggesting that this isoform may be involved in the modulation of gene expression [34]. Recently, in fact, PKC-ζ has been shown to be critical to the activation of the nuclear transcription factor, NF-KB [35]. Further studies on the effects of piroxicam on PKC-ζ, and its role in this NSAID's chemopreventive actions in the colonic malignant transformation process(es) will, therefore, be of interest.

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