1,2-Dimethylhydrazine-induced Altersations in Protein Kinase C Activity in the Rat Preneoplastic Colon

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ABSTRACT

Recently, a number of studies in experimental animals and humans have suggested that alterations in the activity of protein kinase C (PKC) may be involved in the malignant transformation process. To determine whether such alterations in this kinase were present before the development of 1,2-dimethylhydrazine (DMH)-induced colon cancers, rats were given s.c. injections of this procarcinogen (20 mg/kg body weight/week) or diluent for 10 or 15 weeks. Animals were sacrificed after these time periods and colonic epithelium was harvested from each group. The activity and distribution of PKC in the cytosolic and membrane fractions of these preparations as well as 1,2-diacylglycerol mass and phosphoinositide turnover were then examined and compared in the presence and absence of 10 nM 1,25-dihydroxycholecalciferol, an agent which has previously been found to influence these biochemical parameters in the normal rat colonic epithelium.

The results of these studies demonstrate that: (a) the percentage of PKC activity in the membrane fraction was significantly greater in DMH-treated animals compared to their control counterparts at 10 and 15 weeks; (b) the total PKC activity was similar at 10 weeks, but markedly reduced in the colonic mucosa of the DMH-treated group at 15 weeks; (c) 1,2-diacylglycerol mass and phosphoinositide turnover were increased in the colonic mucosa of rats administered this carcinogen at both time points; and (d) in control, but not in DMH-treated animals, in vitro addition of 1,25-dihydroxycholecalciferol increased PKC activity, 1,2-diacylglycerol mass and phosphoinositide turnover at each of the times studied. Based on these findings, it would appear that alterations in PKC activity may play a role in the malignant transformation process of the colon in animals administered DMH.

INTRODUCTION

PKC is a serine/threonine specific protein kinase which is dependent upon phospholipid and calcium (1). This enzyme is ubiquitous in eukaryocytes (2) and appears to play a key role in transmembrane signaling events (1-3). PKC is activated by DAG which is formed in response to extracellular signals by turnover of phosphoinositides (4) as well as other membrane phospholipids (5). This activation process involves translocation of cytosolic PKC to the plasma membrane(s) which, in turn, leads to phosphorylation of target molecules, thereby influencing important cellular processes such as proliferation and/or differentiation (6).

Several lines of evidence have recently been accumulated which indicate that alterations in the activity of this kinase may also be involved in malignant transformation (7-14). Thus, tumor-promoting phorbol esters have been shown to bind to and directly activate PKC (7). Data indicate that PKC may also play a role in the in vitro transformation processes produced by a number of oncogenes (8-10). Cells that overproduce PKC have, moreover, been shown to be more susceptible to transformation by one such activated oncogene, H-ras (11). Additionally, fibroblasts transfected with plasmids containing PKC-complementary DNA, overproduce PKC and demonstrate enhanced tumorigenicity (12, 13). Finally, agents which inhibit PKC have recently been shown to possess in vivo antitumor activity (14). Taken together, these observations would strongly suggest that PKC may be involved in the malignant transformation process(es) of various tissues and organs.

Recent studies in experimental animals and cultured cells (15-18) as well as in humans (19) have, in fact, suggested that alterations in PKC activity may be involved in colonic carcinogenesis. Both bile acids (15, 17, 18) and free fatty acids (16), which may act as promoters of colon cancer (20), have been shown to affect the activity of this kinase. Furthermore, Guillem et al. (19) have recently shown a reduction in PKC activity in human colon adenocarcinomas compared to normal adjacent colonic mucosa.

In this regard, during the past several years, a number of laboratories, including our own, have utilized the DMH model of experimental colon cancer to study the biological characteristics of these tumors (21). DMH-induced neoplasms closely parallel human colon cancer with respect to clinical and pathological features (21). In weekly s.c. doses of 20 mg/kg of body weight, this procarcinogen produces colonic carcinomas in a high percentage of susceptible rodent strains (21-23), with a latency period of approximately 6 months. Moreover, utilizing this model, several laboratories have demonstrated various biochemical changes in rat colonic tissue prior to the development of overt tumors (22-25). While prior investigations have demonstrated early DMH-induced alterations in rat colonic mucosal AMP-dependent protein kinase activity (25), no data are available on the effects of this carcinogen on PKC activity. In the present experiments it was, therefore, of interest to determine whether alterations in PKC, DAG, or phosphoinositides exist in colonic cells of rats administered DMH for 10 and 15 weeks, i.e., before the development of colon cancer. The data from these experiments demonstrate that "premalignant" changes in these biochemical parameters can, indeed, be detected and serve as the basis for this report.

MATERIALS AND METHODS

Animals. Male Sprague Dawley rats weighing 75-100 g were given weekly s.c. injections of diluent or 1,2-dimethylhydrazine dihydrochloride (Sigma Chemical Co., St. Louis, MO) at a dose of 20 mg/kg body weight for 10 and 15 weeks. The stock solution for injections consisted of 400 mg of DMH dissolved in 100 ml of 1.0 M EDTA adjusted to pH 6.5 with sodium hydroxide (23). One week after the last injection, at each time period studied, rat colonic epithelium was isolated accord-
ing to the method of Craven et al. (15) with the following modifications. After ether anesthesia the colon was flushed with 50 ml of Ca- and Mg-free HBSS at 37°C. A 16-gauge needle was placed in the left ventricle and HBSS containing 30 mM EDTA at 37°C was perfused at a flow rate of 20 ml/min for 5-6 min. The colon was then excised, gently everted, and applied to a 5-ml glass pipet attached to a rheostat-controlled electric motor. The everted gut and pipet were then immersed in a 30-ml glass test tube containing cold HBSS. The epithelium was removed by rotating the pipet at 1600 rpm in bursts of 5-10 s for 1-2 min. The epithelium in HBSS was then centrifuged at 500 x g for 5 min and resuspended in oxygenated Krebs-Ringer bicarbonate buffer at pH 7.4. The viability of each preparation was routinely greater than 90% after 2 h, as assessed by Trypan blue exclusion.

Histological Studies. Multiple 1-cm samples (at least 4) were taken from each colon preparation of each group at 10 and 15 weeks and were immediately fixed in 4% formaldehyde. Fixed specimens were then embedded for light microscopic examination and stained with hematoxylin and eosin as previously described by our laboratory (23).

Assay of Protein Kinase C. Colonic preparations from both groups of animals at the 10- and 15-week periods were homogenized by 15 strokes with a motor-driven Teflon pestle in 5 ml of homogenization buffer containing 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 2.0 mM phenylmethylsulfonyl fluoride, 0.5 mM benzimidine, 5.0 mM 2-mercaptoethanol, and 10 mg/liter leupeptin (26). Each homogenate was centrifuged at 100,000 x g for 60 min and the supernatant (cytosolic fraction) was saved. The pelleted was gently resuspended in 5.0 ml of homogenization buffer containing 0.3% Triton X-100 (w/w) and left on ice for 60 min before recentrifugation at 100,000 x g for 60 min. The supernatant (solubilized membrane fraction) was collected and aliquots of both cytosolic and solubilized membrane fractions were assayed for protein (27), using bovine serum albumin as standard, and applied to DEAE-cellulose columns (Poly-prep columns, 200-400 mesh) based on the method of Downes et al. (34). After loading, the columns were eluted serially with 1.5 ml each of (a) water; (b) 50 mM NaCl; (c) 150 mM NaCl; (d) 250 mM NaCl; and counting the radioactivity in a liquid scintillation counter. Routinely, greater than 90% of radioactivity added to the thin layer plates was recovered.

Same-day aliquots of lipid extract were also assayed for 1,2-diacylglycerol mass by the diacylglycerol kinase procedure of Preiss et al. (30) with the following modifications. 3-(N-Morpholinopropane)sulfonic acid buffer, pH 6.6, was substituted for imidazole buffer, pH 6.6 (31). For each reaction tube, DAG kinase (20 million units in 10 μl) was combined with 50 μl of reaction buffer (100 mM sodium 3-(N-morpholino)propane sulfonic acid, pH 6.6, 100 mM NaCl, 25 mM MgCl2, 2 mM EGTA) plus 10 μl of 20 mM dithiothreitol and 10 μl of 10 mM [γ-32P]ATP (5.0 x 10⁶ cpm/nmol), and the mixture was incubated at 25°C for 30 min as described by Wright et al. (32). Duplicate aliquots of diacetyl glycerol standards or cellular lipid extracts were transferred to 75-x 12-mm capped Sarstedt polyethylene tubes and dried under argon. A 20-μl aliquot of resuspension buffer (7.5% octyl-β-D-glucoside, 5 mM cardiolipin, 1 mM diethylstilbestrol pentaacetic acid) was added, vortexed, and sonicated as described (30). To this, 80 μl of the enzyme-ATP mixture were added, followed by vortex mixing and incubation at 25°C for 30 min. The reaction was terminated by the addition of 1.67 ml of CHCl3/methanol/12 N HCl (66:31:1, v/v), followed by 1.67 ml of methanol/H2O/CHCl3 (48:47:3, v/v) as described by MacDonald et al. (31), followed by vortex mixing and centrifugation. The upper phase was removed and discarded, and the lower phase was reextracted with 1.67 ml of methanol/H2O/CHCl3 (48:47:3, v/v). After centrifugation and removal of the upper phase, the labeled phosphatidic acid in the lower phase was assayed directly by scintillation counting of an aliquot as justified by Muldoon et al. (33). Standard curves were generated from the assay of known amounts of 1,2-diacylglycerol and data were expressed as 1,2-diacylglycerol/100 nmol of lipid phosphorus (mol%) (32).
Treatment of Colonic Epithelium with 1,25(OH)2D3. Recent studies by our laboratory (35) have demonstrated that in vitro addition of 1,25(OH)2D3 (10 nm) to normal colonic epithelial preparations rapidly increased phosphoinositide turnover and DAG mass and activated PKC in these preparations. In the present studies, it was, therefore, of interest to determine whether DMH treatment altered the responsiveness of the colonic epithelium to this secosteroid. To address this issue, colonic epithelium obtained from both groups of animals at 10 and 15 weeks was incubated as described above for the various biochemical analyses, followed by the addition of a physiological concentration of 1,25(OH)2D3 (10 nm) or ethanol vehicle (final concentration never exceeding 0.004%) for 1 min. This time and concentration were previously found to result in a maximum response of PKC, DAG, inositol phosphates, and phosphoinositides to this agent (35). The reaction was terminated by the addition of ice-cold buffer and the preparation was processed as described above for analysis of these biochemical parameters.

Statistical Methods. All results are expressed as mean values ± SE. Paired or unpaired t tests were used for all statistical analyses. P < 0.05 was considered significant.

Materials. 1,25(OH)2D3 was kindly provided by Dr. M. R. Uskokovic (Hoffmann-La Roche Inc., Nutley, NJ). Leupeptin, phenylmethylsulfonyl fluoride, histone (type III-S), 1,2-dimethylhydrazine, phosphatidyserine, phosphatidylinositol standards, diethylenetriamine pentaacetic acid, dithiothreitol, ATP, and DEAE-cellulose were purchased from Sigma Chemical Co. (St. Louis, MO). [γ-32P]ATP, [3H]arachidonate, and [3H]myoinositol compounds were obtained from New England Nuclear Research Products (Boston, MA). Octyl-β-d-glucoside was purchased from Boehringer Mannheim (Indianapolis, IN), sn-1,2-diacylglycerol kinase was from Lipidex, Inc. (Westfield, NJ), cardiolipin and sn-dioleoylglycerol were from Avanti Polar Lipids, Inc. (Pelham, AL).

RESULTS

General Observations. In agreement with prior studies by our laboratory (23), at 10 and 15 weeks DMH administration did not significantly affect the weight gain of these animals (data not shown).

Light Microscopic Studies. In agreement with previous studies from our laboratory (22, 23), despite extensive sampling of the colons of each group, no evidence of severe atypia, carcinoma-in-situ, or microscopic carcinomas were seen at 10 and 15 weeks by light microscopy (data not shown). In the present experiments, minimal inflammation was also noted in the colonic preparations which did not differ in intensity after 10 or 15 weeks in each group. These results, therefore, indicate that inflammation per se was not responsible for the biochemical alterations noted in the DMH-treated colonic mucosa (see below).

Colonic Protein Kinase C Activity. After 10 weeks, as shown in Table 1, approximately 82 and 18% of PKC activity was found in the cytosolic and membrane fractions, respectively, of control colonic preparations. In DMH preparations at this time period, however, only 70% of PKC activity was found in the cytosolic fraction with 30% of the activity present in the membrane fraction. Moreover, upon addition of 10 nm 1,25(OH)2D3 to control tissue, PKC was found to significantly translocate from the cytosolic to membrane fraction at 1 min, whereas DMH-treated tissue failed to respond to this agent (Table 1). The total activity of PKC was, however, similar in control and DMH preparations with and without 1,25(OH)2D3 (Table 1).

As can also be seen in this table, after 15 weeks, this pattern of redistribution of PKC activity was further accentuated in the DMH preparations compared to their control counterparts. Furthermore, control tissue again responded to in vitro administration of 1,25(OH)2D3 with translocation of PKC from the cytosol to the membrane fraction, whereas DMH tissue did not respond to this agent. In contrast to the 10-week data, however, the total activity of PKC was not significantly reduced (P < 0.001) in DMH tissue with and without 1,25(OH)2D3 compared to their control counterparts (Table 1). The decrease was seen in cytosolic, not membrane PKC. Background cellular protein kinase activity, in the absence of phosphatidylserine, was 2–4 pmol/mg protein/min and did not change with time, DMH, or 1,25(OH)2D3 treatment.

Colonic DAG Mass. At both 10 and 15 weeks, DMH-treated colonic mucosa was found to possess a significantly greater mass of DAG than control colonic mucosa (Table 2). Moreover, at each of these times, addition of 10 nm 1,25(OH)2D3 led to a significant increase in DAG mass in control, but not in DMH-treated preparations (Table 2).

Colonial Phosphoinositides and Inositol Phosphates. As shown in Table 3, at the 10- and 15-week periods, differences were noted in the incorporation of [3H]myoinositol into individual phosphoinositides and inositol phosphates between control and DMH-treated preparations. As can be seen in this table, the turnover of labeled phosphatidylinositol derivatives, represented by the ratio of total labeled inositol phosphates/total labeled phosphoinositides was significantly greater at both time periods in DMH-treated preparations compared to their control counterparts. The majority of the change in inositol phosphate levels was due to changes in IP with no significant change noted in the levels of the second messenger, IP3. Finally, control, but not DMH preparations, were found to significantly increase this ratio and IP3 levels after in vitro addition of 10 nm 1,25(OH)2D3 for 1 min (Table 3).

DISCUSSION

The present results demonstrate for the first time that alterations in PKC activity could be detected in the preneoplastic colons of rats after administration of the chemical carcinogen DMH for 10 and 15 weeks. At these time points, despite extensive histological sampling of the entire colon in these animals, no evidence of inflammation, severe atypia, carcinoma in situ, or microscopic adenocarcinomas were evidenced by light microscopic examination.

At 10 weeks, DMH-treated rats had a different cellular distribution of PKC activity, with a greater percentage in the membrane fraction than in their control counterparts. No differences, however, were noted in the total activity of this enzyme in the colon of these two groups at this time. Previous studies have demonstrated that PKC activity is elevated in the particulate fraction of cells under a variety of conditions, including phorbol ester tumor promotion, viral transformation, and rapid cell growth (reviewed in Ref. 36). Based on these findings, it has been suggested that changes in the distribution of PKC may serve as a regulatory mechanism to alter the endogenous substrates available for phosphorylation by this kinase, thereby leading to tumor promotion and malignant transformation (36).

At 15 weeks, the redistribution of PKC seen in the DMH colonocytes at 10 weeks was further accentuated and, moreover, the total activity in DMH-treated colonic mucosa was reduced to approximately one-half of the activity present in control colonic mucosa. It is important to note that changes in crude PKC activity levels may reflect alterations in the expression of endogenous PKC inhibitors of phosphatases, both of which might interfere with the histone phosphorylation assay. Purification on DEAE-cellulose, however, likely removes the majority of interfering compounds. Of interest, total PKC activity in
DMH-INDUCED ALTERATIONS IN COLONIC PKC

Table 1 PKC cellular distribution and total activity in colonic epithelium of control and DMH-treated rats after 10 and 15 weeks*

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Membrane-associated activity (pmol/mg protein/min)</th>
<th>Total activity (pmol/mg protein/min)</th>
<th>Membrane-associated activity (pmol/mg protein/min)</th>
<th>Total activity (pmol/mg protein/min)</th>
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<tbody>
<tr>
<td></td>
<td>10 wk</td>
<td>15 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.8 ± 0.5 (18.0 ± 2.5)</td>
<td>20.9 ± 0.8</td>
<td>6.2 ± 0.7 (23.0 ± 2.6)</td>
<td>27.0 ± 2.5</td>
</tr>
<tr>
<td>DMH</td>
<td>5.3 ± 0.4 (30.0 ± 2.1)*</td>
<td>17.7 ± 0.5</td>
<td>6.3 ± 0.9 (42.0 ± 6.2)*</td>
<td>15.1 ± 2.1*</td>
</tr>
<tr>
<td>Control + 1,25(OH)2D3</td>
<td>6.0 ± 0.3 (32.0 ± 1.5)*</td>
<td>18.9 ± 1.2</td>
<td>12.4 ± 0.7 (42.0 ± 2.4)*</td>
<td>29.6 ± 1.6</td>
</tr>
<tr>
<td>DMH + 1,25(OH)2D3</td>
<td>5.0 ± 0.2 (29.0 ± 1.0)</td>
<td>17.2 ± 2.5</td>
<td>7.5 ± 0.7 (52.0 ± 5.1)</td>
<td>14.4 ± 1.0</td>
</tr>
</tbody>
</table>

* Values represent means ± SE of number of separate animals analyzed at each time point. Values in parentheses, percentage of total activity.

Table 2 DAG mass in colonic epithelium of control and DMH-treated rats after 10 and 15 weeks*

<table>
<thead>
<tr>
<th>Preparations</th>
<th>10 wk</th>
<th>15 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.36 ± 0.01</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>DMH</td>
<td>0.41 ± 0.01*</td>
<td>0.44 ± 0.01*</td>
</tr>
<tr>
<td>Control + 1,25(OH)2D3</td>
<td>0.53 ± 0.01*</td>
<td>0.62 ± 0.01*</td>
</tr>
<tr>
<td>DMH + 1,25(OH)2D3</td>
<td>0.40 ± 0.01</td>
<td>0.44 ± 0.01</td>
</tr>
</tbody>
</table>

* Values represent means ± SE of number of separate animals analyzed at each time period.

The exact significance of subsequent elevations in DAG, PKC translocation, and down-regulation to colonic malignant transformation in this model remains unclear. The presence of these changes in the muscosa prior to overt neoplasia, however, suggests that they may play a role in the early stage(s) of the multistage malignant transformation process. Additional factors would then be necessary for the development of carcinoma in certain of these cells.

In the present experiments, DAG mass was found to be increased in the colonic mucosa of rats treated with DMH for 10 and 15 weeks. It would, therefore, appear reasonable to suggest that elevations in DAG might underlie the translocation of PKC activity to the membrane at these time periods. While the origin of these DMH-induced elevations in DAG levels is not totally clear, based on our study of colonic phosphoinositide turnover in these animals, it would appear that at least a portion of this DAG may come from the breakdown of these membrane phospholipids. While prior studies in oncogene-transformed cells have also suggested that phosphoinositides may serve as the source for increased DAG, others have suggested that lipid activator of PKC was derived from the breakdown of other membrane phospholipids. It is, therefore, possible that the elevations in DAG induced DMH in the present experiments were also derived from these other phospholipids such as phosphatidylycholine and phosphatidylethanolamine as well as conceivably from phosphatidic acid.

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Down-regulation in phorbol-treated and ras-transfected cells appears to be caused by proteolytic cleavage of PKC molecules. Furthermore, cleavage of PKC has been associated with the appearance of a protein kinase in the cytosol which was independent of the activators of PKC, i.e., calcium, phosphatidylyserine and DAG (42). While the physiological significance of this protein kinase found by cleavage of PKC remains unclear, Chida et al. (42) have suggested that it may diffuse into the cytoplasm and nucleus of the cell and therefore transduce signals from the cytosolic side of the plasma membrane to the nucleus, thereby inducing malignant transformation. Based on studies which have demonstrated synergism between the actions of ras oncogenes and phorbol esters on cellular transformation (43), Weyman et al. (8) have also recently speculated that down-regulation of PKC, or a process closely linked to this phenomenon, may be important in the multistage carcinogenesis process. In contrast to these hypotheses, Wolfman et al. (9) have suggested that down-regulation of PKC may actually represent an unsuccessful attempt by transformed cells to negatively modulate proliferation signals generated by oncogene products. The exact relationship between down-regulation of PKC noted in the current experiments to malignant transformation, therefore, remains unclear.

In agreement with prior studies from our laboratory (35), in the present experiments 1,25(OH)2D3 was found to stimulate colonic mucosal turnover of phosphoinositides, increase DAG.
mass, and activate PKC in control animals. In DMH-treated rats, however, this secosteroid failed to significantly alter any of these biochemical parameters in the rat colon. This is of interest, since it would indicate that DMH-treated neoplastic colonic cells have already lost their responsiveness to this agent, well before the development of overt tumors. While the exact mechanism(s) responsible for this loss of responsiveness to 1,25(OH)2D3 is also unclear at this time, prior studies in oncogene-transformed cells have demonstrated a similar loss of response to various growth factors, secondary to a variety of alterations involving the inositol phospholipid signal transduction pathways and/or PKC (44–46). It should also be noted that this secosteroid has been shown to possess antiproliferative actions in cell lines derived from human colon cancers (47, 48). It is, therefore, conceivable that this loss of responsiveness to 1,25(OH)2D3 may also contribute to the development of DMH-induced colon cancers.

Recently, Craven and DeRubertis (49) have demonstrated differences in the subcellular distribution of PKC in rat colonic cells with different proliferative activities. Since DMH is known to stimulate colonic epithelial cell proliferation (50), it is possible that the PKC alterations noted in the present experiments were secondary to proliferative changes induced by this carcinogen. In this regard, however, prior studies in this model, using an identical treatment regimen, have demonstrated comparable increases in proliferation induced by DMH after 10 and 15 weekly injections (50). It is, therefore, difficult to reconcile changes in proliferation per se with the sequential changes noted in PKC subcellular distribution and total activity in the present experiments. Moreover, in agreement with previous studies utilizing the phorbol ester 12-O-tetradecanoylphorbol-13-acetate as well as deoxycholate (49), in preliminary experiments performed by our laboratory, in vitro addition of 1,25(OH)2D3 was found to activate PKC to a similar extent in proliferative and nonproliferative normal colonic epithelial cells. Taken together, these observations, therefore, strongly suggest that DMH-induced alterations in cellular proliferation are unlikely to explain all the effects of this agent on PKC activity found in the current studies.

In summary, the present results have demonstrated alterations in PKC activity, DAG mass, and phosphoinositide turnover in the preneoplastic colons of rats administered DMH for 10 to 15 weeks. Furthermore, in vitro addition of 1,25(OH)2D3 failed to elicit the normal pattern of response of these biochemical parameters in the colons of carcinogen-treated animals. Based on these observations, it would appear that changes in PKC activity may play a role in the early stages of the malignant transformation process induced by DMH. Further studies concerning this possible relationship are now being conducted in this experimental model of colonic adenocarcinoma by our laboratory.

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4. Unpublished observations.


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