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Carcinogens stimulate phosphorylation of ethanolamine derived from increased hydrolysis of phosphatidylethanolamine in $C_3H/101/2$ fibroblasts

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Many human tumors contain high concentrations of ethanolamine phosphate (EtnP). An important question is whether increased formation of EtnP is merely the consequence of cell transformation, or is it associated with the process of carcinogenesis. Here we show that in $C_3H/10T1/2$ embryonic fibroblasts, an established cellular model for the study of carcinogenesis, the environmental carcinogens, 7,12-dimethylbenz[a]anthracene (DMBA) and benzo[a]pyrene (B[a]P) (0.1–1 μg/ml concentration; 24 h treatment), stimulate phosphorylation of ethanolamine derived from increased hydrolysis of phosphatidylethanolamine. The results suggest that increased formation of EtnP is associated with the early stages of carcinogenesis. This observation may have prognostic value.

Ethanolamine phosphate; Phosphatidylethanolamine; 7,12-Dimethyl-benz[a]anthracene; Benzo[a]pyrene; Fibroblast

1. INTRODUCTION

It is now well established that most human tumors contain high concentrations of phosphomonoesters, including ethanolamine phosphate (EtnP) and choline phosphate (ChoP) [1–8]. Interestingly, in several different types of tumors the major phosphomonoester has been identified as EtnP [9–14]. These studies raise the following important questions: (i) what mechanism(s) is (are) responsible for the increased levels of phosphomonoesters in cancer cells?; (ii) is increased formation of phosphomonoesters merely the consequence of cell transformation, or is this process associated with, or does it perhaps contribute to, the transformation process?; (iii) what is the mechanism of selective enhancement of EtnP in the respective cancer cells?

The embryonic $C_3H/10T1/2$ fibroblast line is a well-established model system for the study of chemical carcinogenesis [15–17]. Our hypothesis has been that if increased formation of phosphomonoesters is associated with the process of cell transformation, then in this cell line carcinogens should enhance the cellular level of EtnP and/or ChoP at a relatively early stage of treatment. Here we show that treatment of these fibroblasts with the environmental carcinogens, 7,12-dimethylbenz[a]anthracene (DMBA) or benzo[a]pyrene (B[a]P), for 24 h enhanced the formation of EtnP, but not ChoP. The results presented here indicate that this carcinogen effect is due, in part, to increased activity of an ethanolamine (Etn) kinase. Two different Etn pools, derived from the external medium and from phosphatidylethanolamine (PtdEtn), are likely to serve as substrates for Etn kinase.

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Abbreviations: Etn, ethanolamine; EtnP, ethanolamine phosphate; ChoP, choline phosphate; PtdEtn, phosphatidylethanolamine; PLD, phospholipase D; PLC, phospholipase C; DMBA, 7,12-dimethylbenz[a]anthracene; B[a]P, benzo[a]pyrene.

2. EXPERIMENTAL

2.1. *Materials*

DMBA, B[a]P, and Dowex-50W (H^+ form) were purchased from Sigma; [2-^{14}C]ethanolamine (50 mCi/mmol) and [methyl-^{14}C]choline were brought from Amersham; tissue culture reagents were from Gibco BRL.

2.2. *Cell culture*

The $C_3H/10T1/2$ embryonal fibroblast line was from American Type Culture Collection. Fibroblast cultures were maintained in basal Eagle's medium supplemented with 10% fetal calf serum (heat-inactivated). Cells were used between passages 10–15.

2.3. *Determination of EtnP formation and phospholipase-mediated hydrolysis of PtdEtn in fibroblasts*

$C_3H/10T1/2$ fibroblasts were grown in 6-well culture dishes for 48 h (up to ~ 80% confluency) in the presence of [^{14}C]Etn (1.0 μCi/ml). In the experiment shown in Fig. 1, carcinogens were present during the last 24 h of the labeling period at concentrations indicated in the figure legend. For the determination of cellular content of [^{14}C]Etn metabolites, cells were washed twice with 5 ml medium followed by the addition of ice-cold methanol (2 ml) to the wells, and by rapid transfer of methanol extracts to tubes containing chloroform. After phase separation, the water-soluble derivatives of [^{14}C]Etn were separated on Dowex-50[H^+] columns as previously described [18]. [^{14}C]Etn and its metabolites were identified by thin-layer chromatography [19]. PtdEtn was separated from other phospholipids as previously indicated [19].

For the determination of PLD- and PLC-mediated hydrolysis of PtdEtn, fibroblasts were labeled with [^{14}C]ethanolamine as above but the carcinogens were added for the last 20 h of the labeling period. Cells were washed and then incubated in fresh medium for 4 h (to decrease the background levels of water-soluble [^{14}C]Etn products; see [20]) in the absence or presence of carcinogens. Cells were washed again, and then incubated for 30–120 min in 1.0 ml medium containing 2 mM unlabeled Etn. We have established earlier that in fibroblasts unlabeled Etn can effectively block metabolism of newly formed [^{14}C]Etn [20,21] and [^{14}C]EtnP [18], and that [^{14}C]PtdEtn is the common precursor for newly formed [^{14}C]Etn and [^{14}C]EtnP (formed by PLD- and PLC-mediated mechanisms, respectively). Ice-cold methanol (2 ml) was added to the wells (without removing the medium), and [^{14}C]Etn and [^{14}C]EtnP were determined as described above.

2.4. *Determination of cellular uptake and phosphorylation of [^{14}C]Etn in fibroblasts*

C_3H/10T1/2 fibroblasts, grown in 6-well tissue culture dishes up to ~80% confluency, were treated with B[a]P (0.5 μg/ml) or DMBA (0.5 μg/ml) for 24 h, followed by incubation of cells in the presence of [2-^{14}C]Etn (810,000 dpm/well) for 30–120 min. Cells were washed (for 15 s) twice with 5 ml medium, followed by the addition of 2 ml ice-cold methanol. Water-soluble [^{14}C]Etn metabolites and [^{14}C]PtdEtn were separated as described above.

3. RESULTS AND DISCUSSION

When cells are labeled with [^{14}C]Etn for an extended time period (presently for 48 h), the different metabolic pools of [^{14}C]PtdEtn derivatives are in near isotopic equilibrium. As a consequence, changes in the ^{14}C content of various Etn-containing compounds will reflect proportional changes in their mass. This radiolabeling method is sensitive and is also suitable for the determination of the mechanisms involved in the metabolism of Etn-containing compounds. Therefore, in the present study, we used mostly [^{14}C]Etn-labeled fibroblasts to determine possible effects of carcinogens on the cellular levels of PtdEtn and its water-soluble metabolites.

Chronic (24 h) treatment of [^{14}C]Etn-labeled fibroblasts with either B[a]P or DMBA decreased the cellular level of [^{14}C]PtdEtn. A detectable decrease (8–10%) in [^{14}C]PtdEtn content was elicited by 0.1 μg/ml concentration of carcinogens (a suboptimal concentration with respect to carcinogenesis; [15]), while maximal effects (17 and 20% decrease) required a 1 μg/ml concentration of B[a]P and DMBA, respectively (Fig. 1A). In order to verify that the observed reduction in cellular [^{14}C]PtdEtn indeed corresponded to reduced phospholipid mass, we next determined the P_i content of PtdEtn in fibroblasts treated with 0.5 μg/ml of DMBA for 24 h. In these experiments special care was taken to minimize errors which could result from slight differences in the initial cell number. Therefore, DMBA was added to fibroblasts at ~75% confluency, so that at the time of harvest the fibroblast cultures were in the confluent state (i.e. in each 150-mm dish, the cell number was the same). In agreement with our expectation, DMBA reduced the P_i content of PtdEtn from 2.35 ± 0.16 μg/dish (n = 8) to 1.86 ± 0.14 μg/dish (n = 8).

In each case, the carcinogen-induced decrease in the

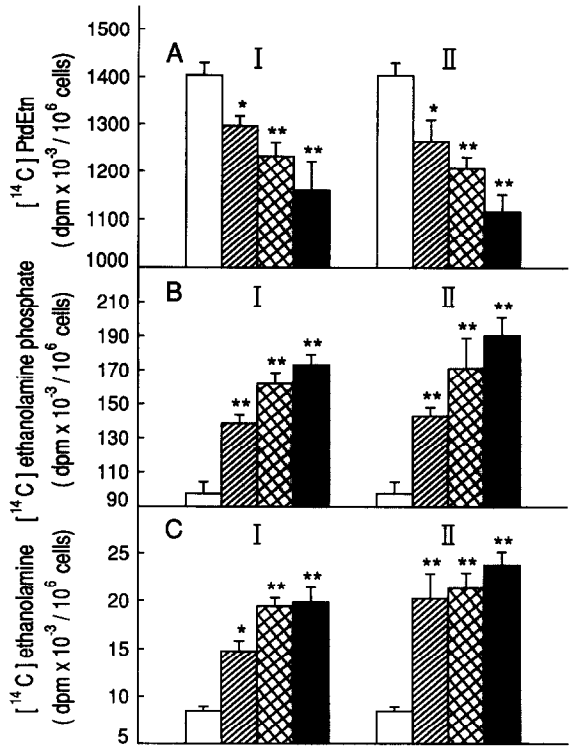

Fig. 1. Effects of carcinogens on the cellular content of [^{14}C]PtdEtn, [^{14}C]EtnP and [^{14}C]Etn in C_3H/10T1/2 fibroblasts. Fibroblasts, grown in 6-well culture dishes, were labeled with [^{14}C]Etn for 48 h. Carcinogen was not present (□), or B[a]P (I) or DMBA (II) was added to fibroblasts at the concentrations of 0.1 μg/ml (□), 0.5 μg/ml (□), or 1 μg/ml (■) for the last 24 h of the labeling period. The cellular content of [^{14}C]PtdEtn (A), [^{14}C]EtnP (B), and [^{14}C]Etn (C) was determined as described in section 2. Data are the mean ± S.E.M. of three experiments each performed in triplicate. *-**Significantly ($P < 0.05$*–0.01**) different from the respective control (Student's t-test).

cellular [^{14}C]PtdEtn level was accompanied by a pronounced increase in the cellular level of [^{14}C]EtnP (Fig. 1B) and by a moderate elevation in the cellular content of [^{14}C]Etn (Fig. 1C). In contrast, these carcinogens failed to enhance the formation of glycerophosphoethanolamine (data not shown). Treatment of fibroblasts with either carcinogen (1 μg/ml) for only 4 h did not elicit statistically significant changes in the cellular level of any of the above Etn-containing compounds.

Similar experiments were performed with [^{14}C]-choline-labeled fibroblasts. While carcinogens (1 μg/ml; 24 h treatment) decreased the cellular content of [^{14}C]PtdCho about 8–10%, we have not observed corresponding increases in the cellular levels of [^{14}C]choline and/or [^{14}C]ChoP (data not shown). This would be consistent with increased PLD-mediated degradation of PtdCho in carcinogen-treated fibroblasts, followed by the efflux of newly formed [^{14}C]choline. Experiments are presently underway in our laboratory to address this and other possibilities.

Carcinogen-induced increase in the cellular levels of

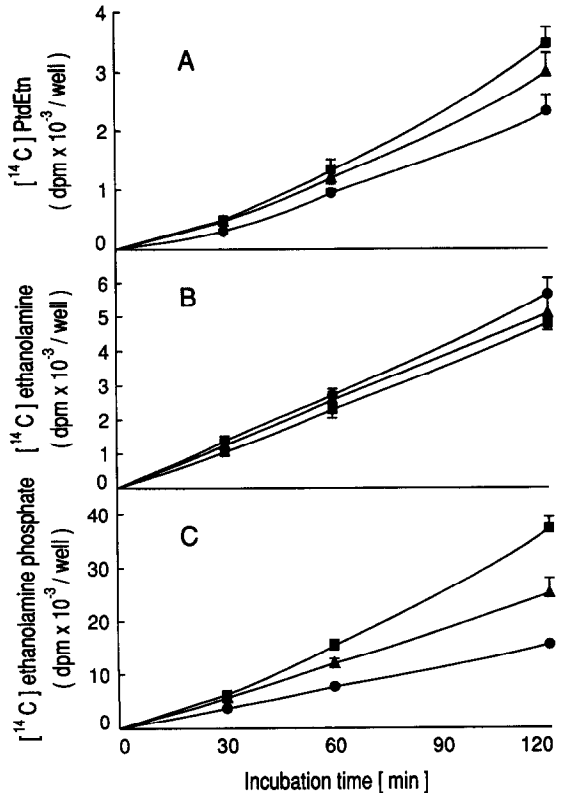

Fig. 2. Effects of carcinogens on the uptake and metabolism of [^{14}C]Etn in C_3H/10T1/2 fibroblasts. Fibroblasts, grown in 6-well culture dishes, were untreated (●), or were treated with 0.5 μg/ml of B[a]P (▲) or 0.5 μg/ml of DMBA (■) for 24 h, followed by incubation of fibroblasts in the presence of [^{14}C]Etn (810,000 dpm/well) for up to 120 min. Incorporation of exogenous [^{14}C]Etn into the cellular pools of PtdEtn (A), Etn (B), and EtnP (C) were determined as described in section 2. Each point represents the mean ± S.E.M. of three experiments each performed in quadruplicate.

[^{14}C]EtnP and [^{14}C]Etn could reflect an inhibition of PtdEtn synthesis, but also could be due to increased hydrolysis of [^{14}C]PtdEtn. Alternatively, carcinogens could enhance cellular uptake and/or phosphorylation of [^{14}C]ethanolamine. To distinguish among these, not necessarily exclusive, mechanisms, we next examined the effects of carcinogens on the cellular uptake and phosphorylation of [^{14}C]Etn, as well as on the synthesis of PtdEtn from this precursor. Pretreatment of fibroblasts with 0.5 μg/ml of B[a]P or DMBA for 24 h resulted in enhanced labeling of PtdEtn with [^{14}C]Etn during a subsequent labeling period (Fig. 2A). At each time interval (30–120 min) of the labeling period examined, B[a]P and DMBA stimulated incorporation of [^{14}C]Etn into PtdEtn 1.3- to 1.5-fold and 1.4- to 1.6-fold, respectively. During the same incubation period, carcinogen-pretreated cells accumulated somewhat less [^{14}C]Etn (Fig. 2B), but they formed significantly more [^{14}C]EtnP than control cells (Fig. 2C). In each case, the carcinogen-induced increase in [^{14}C]EtnP formation corresponded to the formation of [^{14}C]PtdEtn. These experiments established that carcinogens do not inhibit PtdEtn synthesis. Also, the results made it clear that under the present conditions [^{14}C]EtnP could not possibly be formed from [^{14}C]PtdEtn, because the ^{14}C content of this latter compound was too small to serve as a precursor. Finally, since carcinogens decreased, rather than enhanced, the cellular level of [^{14}C]Etn, it appeared that the stimulatory effects of carcinogens on the cellular content of [^{14}C]EtnP were due to increased phosphorylation of [^{14}C]Etn, followed by a compensatory increase in the cellular uptake of [^{14}C]Etn.

While the above experiments indicated that carcinogens can enhance the formation of EtnP by a mechanism which is independent of PtdEtn hydrolysis, the question whether the observed carcinogen-induced loss of PtdEtn (Fig. 1A) and increase in cellular EtnP level (Fig. 1B) were causally related processes or not remained to be established. To clarify this issue, we next determined the effects of carcinogens on PLC- and PLD-mediated hydrolysis of PtdEtn. As shown in Table I, treatment of [^{14}C]Etn-labeled fibroblasts with carcinogens for 2 h failed to enhance the formation of [^{14}C]EtnP from the cellular [^{14}C]PtdEtn substrate pool. In contrast, treatments of labeled fibroblasts with B[a]P (0.5 μg/ml) or DMBA (0.5 μg/ml) similarly enhanced the formation of [^{14}C]Etn from [^{14}C]PtdEtn (Fig. 3). We should emphasize here that addition of 2 mM Etn to the incubation medium prevented metabolism of newly formed [^{14}C]Etn and [^{14}C]EtnP [18,20,21]. For this reason, and because there was an inverse relationship between carcinogen-induced formation of [^{14}C]Etn and the loss of cellular [^{14}C]PtdEtn (data not shown), formation of [^{14}C]Etn most probably occurred by PLD-mediated hydrolysis of [^{14}C]PtdEtn. On the other hand, when the above experiment (Fig. 3) was repeated so that the incubation medium did not contain unlabeled Etn, about 95% of [^{14}C]PtdEtn-derived water-soluble ^{14}C activity was present in EtnP. Thus, under normal conditions (i.e. in the absence of unlabeled Etn), PtdEtn hy-

Table I

Effects of carcinogens on the formation of [^{14}C]EtnP from the prelabeled cellular pool of [^{14}C]PtdEtn in C_3H/10T1/2 fibroblasts

Treatment with	Formation of [^{14}C]EtnP (dpm/10^6 dpm of [^{14}C]PtdEtn/2 h)
None	43,060 ± 2,940
B[a]P	45,180 ± 4,410
DMBA	37,720 ± 3,780

Fibroblasts, grown in 6-well culture dishes, were labeled with [^{14}C]Etn for 48 h and then treated with 0.5 μg/ml of B[a]P or DMBA for the last 20 h of the labeling period, as indicated. Fibroblasts were washed and then incubated for 4 h in fresh medium in the absence or presence of carcinogens (0.5 μg/ml), as appropriate. Fibroblasts were washed again and then incubated for 2 h in fresh medium (containing 2 mM unlabeled Etn) to determine formation of [^{14}C]EtnP. Data are the mean ± S.E.M. of two experiments each performed in triplicate.

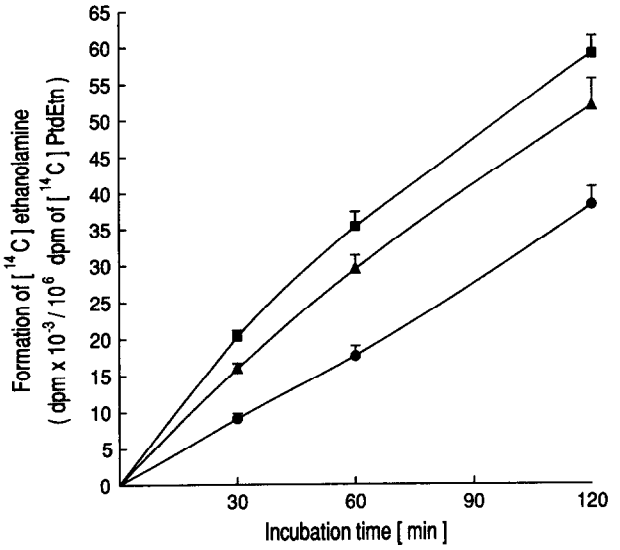

Fig. 3. Effects of carcinogens on the formation of [^{14}C]Etn from the pre-labeled cellular pool of [^{14}C]PtdEtn in C_3H/10T1/2 fibroblasts. Fibroblasts, grown in 6-well culture dishes and labeled with [^{14}C]Etn for 48 h, were untreated (●) or were treated with 0.5 μg/ml of B[a]P (▲) or 0.5 μg/ml of DMBA (■) for the last 20 h of the labeling period. Fibroblasts were washed and then incubated for 4 h in fresh medium in the absence or presence of carcinogens (0.5 μg/ml) as appropriate. Fibroblasts were washed again, and then incubated for up to 120 min in fresh medium (containing 2 mM unlabeled Etn) to determine formation of [^{14}C]Etn. Each point represents the mean ± S.E.M. of three experiments, each performed in triplicate.

drolysis in carcinogen-treated cells yields practically only EtnP.

In other experiments, carcinogen-induced formation of [^{14}C]Etn from [^{14}C]PtdEtn was not affected by 1–10 mM choline or 1-serine, which argues against stimulation of a base-exchange mechanism by carcinogens. However, further experiments are required to definitely prove activation of PLD in carcinogen-treated cells.

In summary, the results presented here show that in embryonic fibroblasts carcinogens stimulate the formation of EtnP well before transformation of fibroblasts occurs. Stimulation of an Etn kinase activity appears to play a key role in this process. Since blood Etn levels are low [22], it is reasonable to assume that PtdEtn-derived Etn also serves as a substrate for Etn kinase. These data raise the possibility that determination of EtnP levels may help to better identify the early stages of carcinogenesis. Further experiments are required to clarify whether the presently observed changes in the cellular levels of EtnP and PtdEtn play a role in carcinogenesis.

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