Wortmannin inhibits carcinogen-stimulated phosphorylation of ethanolamine and choline

Zoltan Kiss*, Masahiro Tomono

The Hormel Institute, University of Minnesota, 801 16th Avenue NE, Austin, MN 55912, USA

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Abstract We have previously reported that in C3H/10T1/2 fibroblasts the environmental carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) stimulated phosphorylation of ethanolamine (Etn). Here we show that in these fibroblasts DMBA also stimulates phosphorylation of choline (Cho). Wortmannin (50–200 nM), an established inhibitor of phosphatidylinositol-3-kinase (PI3K), significantly inhibited DMBA-induced phosphorylation of both Etn and Cho. Wortmannin also inhibited the effect of insulin, a major activator of PI3K, on DNA synthesis. However, insulin had no effect on the phosphorylation of Etn and Cho. These data suggest that a carcinogen-induced kinase phosphorylates both Etn and Cho, and that the inhibitory effect of wortmannin on Etn/Cho kinase activity may be unrelated to its inhibitory effect on PI3K activity.

Key words: 7,12-Dimethylbenz[a]anthracene; Ethanolamine; Choline; Kinase; Wortmannin; Phosphatidylinositol-3-kinase

1. Introduction

Virtually all human tumors examined so far contain high concentrations of phosphomonoesters mostly represented by either ethanolamine phosphate (EtnP) or choline phosphate (ChoP), or both (referenced in [1]). This suggests a causal relationship between high cellular levels of phosphomonoesters and altered cell growth. Two recent observations further strengthen this possibility. Thus, Cuadrado et al. [2] recently reported that formation of ChoP represents a key step during mitogenic stimulation of cells by several growth factors. We also reported [1] that in C3H/10T1/2 fibroblasts, a frequently used cellular model of chemical carcinogenesis [3–5], chemical carcinogens relatively rapidly stimulated both Etn formation from phosphatidylethanolamine (PtdEtn), and subsequent phosphorylation of Etn by a kinase activity. This latter finding is of considerable interest because it may not only reveal a possible role for EtnP in cell growth regulation, but it may also have prognostic value. For these reasons we have a continuous interest in the characterization of carcinogen effects on the phosphorylation of Etn and Cho.

When fibroblasts were labeled with [^{14}C]Etn or [^{14}C]Cho for 48 h, followed by treatments of subconfluent cultures with 7,12-dimethylbenz[a]anthracene (DMBA) for the last 24 h of the labeling period, we observed significant carcinogen-induced increases in the cellular level of [^{14}C]EtnP, but not that of [^{14}C]ChoP [1]. This could mean that these cells possessed a carcinogen-inducable Etn-specific kinase activity. However, carcinogens also stimulated PtdEtn hydrolysis while they decreased PtdCho hydrolysis [6]. Thus, it was also possible that a carcinogen-stimulated kinase phosphorylates both Etn and Cho, but it preferentially uses substrates, presently Etn, derived from increased phospholipid hydrolysis. Finally, the Etn- and Cho-phosphorylating system(s) may respond to carcinogens differently when fibroblasts are present in the growing or non-growing phases. In order to distinguish among these possibilities, here we compared the effects of carcinogen treatment on the phosphorylation of Cho and Etn in confluent cultures. The results clearly indicate that in these fibroblasts, DMBA has comparable stimulatory effects on the phosphorylation of ^{14}C-labeled Etn and Cho added to the medium.

Wortmannin is thought to be a specific inhibitor of phosphatidylinositol-3-kinase (PI3K) [6–8]. Here we also examined the effects of wortmannin on the phosphorylation of Etn and Cho. The results show that wortmannin inhibits phosphorylation of both Etn and Cho.

2. Experimental

2.1. Materials

DMBA, wortmannin, Etn and Dowex-50W (H^+ form) were purchased from Sigma; insulin was from Boehringer Mannheim; [2-^{14}C]ethanolamine (55 mCi/mmol), [*methyl*-^{14}C]choline (50 mCi/mmol) and [*methyl*-^{3}H]thymidine (85 mCi/mmol) were bought from Amersham; tissue culture reagents were from Gibco-BRL.

2.2. Cell culture

The C3H/10T1/2 embryonal fibroblast line was from American Type Culture Collection and was maintained in basal Eagle's medium supplemented with 10% fetal calf serum (heat-inactivated). Cells were used between passages 10–14. The NIH 3T3 clone-7 fibroblast line was kindly provided by Dr. Douglas R. Lowy (National Cancer Institute, National Institutes of Health, Bethesda, Maryland, U.S.A.). NIH 3T3 fibroblasts were continuously cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal calf serum, penicillin (50 units/ml)/streptomycin (50 μl/ml), and glutamine (2 mM).

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

Fibroblasts were grown in 12-well tissue culture dishes to confluency and then either remained untreated or were treated with DMBA (0.5 μg/ml) for 24 h. When indicated, wortmannin was added for the last hour of incubation. This was followed (with no washing step being included) by incubations of fibroblasts for 1–3 h in the presence of 50 μM [^{14}C]Etn (802,000–991,000 dpm/well) or 50 μM [^{14}C]Cho ($1.23 \times 10^6 - 1.28 \times 10^6$ dpm/well). At the conclusion of incubations, the incubation medium was aspirated, then fibroblasts were rapidly (within 20 s) washed with 5 ml medium followed by the addition of ice-cold methanol (2 ml) to the wells; this was followed by scraping of cells into methanol, and by rapid transfer of methanol extracts to tubes containing chloroform. After phase separation, the water-soluble derivatives of [^{14}C]Etn and [^{14}C]Cho were separated on Dowex-50W (H^+) columns

*Corresponding author. Fax: (1) (507) 437 9606.

Abbreviations: Etn, ethanolamine; EtnP, ethanolamine phosphate; Cho, choline; ChoP, choline phosphate; DMBA, 7,12-dimethylbenz[a]anthracene; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PI3K, phosphatidylinositol-3-kinase.

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as previously described [6]. The ^{14}C activity in phospholipids was determined as previously indicated [6].

2.4. Labeling of cellular DNA with [^3H]thymidine

NIH 3T3 fibroblasts were grown in 12-well tissue culture dishes to about 40–50% confluency in the presence of 10% serum. This was followed by incubation of fibroblasts in serum-free medium for 24 h, and then by treatments with insulin and/or wortmannin for 16 h. Finally, incubations were continued in the presence of [*methyl*-^3H]thymidine (5 μCi/ml) for 60 min. Fibroblasts were washed twice with phosphate-buffered saline and then four times with 5% trichloroacetic acid. The acid-insoluble material was redissolved in 0.3 M sodium hydroxide, and an aliquot was taken to measure DNA-associated ^3H-activity in a liquid scintillation counter.

3. Results

In a previous work, the effects of carcinogens on the phosphorylation of Etn were examined in subconfluent cultures of C3H/10T1/2 fibroblasts. In these fibroblasts, phosphorylation of Etn occurred at a relatively high rate and an optimal concentration (0.5 μg/ml) of DMBA enhanced EtnP formation about 2-fold [1]. Since then, we performed a more detailed examination of phosphorylation of Etn at various stages of confluency. In unstimulated confluent cultures, the rate of Etn phosphorylation was lower than in subconfluent cultures, but carcinogens elicited significantly greater stimulation of Etn kinase activity in the former cells. Thus, in this work the effects of DMBA and wortmannin on the phosphorylation of Etn and Cho were determined in confluent cultures.

When confluent C3H/10T1/2 fibroblasts were incubated in the presence of [^{14}C]Etn, in untreated fibroblasts (A) and in fibroblasts treated with DMBA (0.5 μg/ml) for 24 h (B), the increase in the cellular content of [^{14}C]Etn reached a plateau after 1–2 h incubation. During the first hour of incubation, 50–200 nM concentrations of wortmannin appeared to slightly enhance the cellular content of [^{14}C]Etn (Fig. 1A and 1B), but this compound clearly had no inhibitory effect at any time interval examined. Untreated fibroblasts accumulated about twice more [^{14}C]EtnP (Fig. 1C) than [^{14}C]Etn (Fig. 1A) by the end of the 3 h incubation period. Although in this presentation it is somewhat difficult to observe, 50–200 nM concentrations of wortmannin caused 16–28% inhibition of [^{14}C]EtnP formation in non-DMBA-treated fibroblasts (Fig. 1C); this small inhibition was reflected in decreased ^{14}C-labeling of PtdEtn (Fig. 1E). In DMBA-treated fibroblasts (Fig. 1D) the rate of [^{14}C]EtnP formation at various incubation times was 4.2- to 6.4-fold greater compared to untreated fibroblasts (Fig. 1C). 200 nM Wortmannin inhibited [^{14}C]EtnP formation by 24, 45 and 51% after 1, 2 and 3 h incubation, respectively (Fig. 1D). It should be noted that 50 nM wortmannin was almost as effective as 200 nM wortmannin in inhibiting phosphorylation of Etn (Fig. 1D), while an increase of concentration of wortmannin above 200 nM did not cause additional inhibition (data not shown). Despite the large increase in [^{14}C]EtnP formation in DMBA-treated fibroblasts, incorporation of [^{14}C]Etn into PtdEtn was only slightly different in the untreated (Fig. 1E) and DMBA-treated fibroblasts (Fig. 1F). Also, wortmannin similarly inhibited the formation of [^{14}C]PtdEtn in the untreated and DMBA-treated fibroblasts.

The effect of DMBA on the metabolism of [^{14}C]Cho was very similar to its effect on [^{14}C]Etn metabolism. Thus, both in the untreated (Fig. 2A) and DMBA-treated fibroblasts (Fig. 2B),

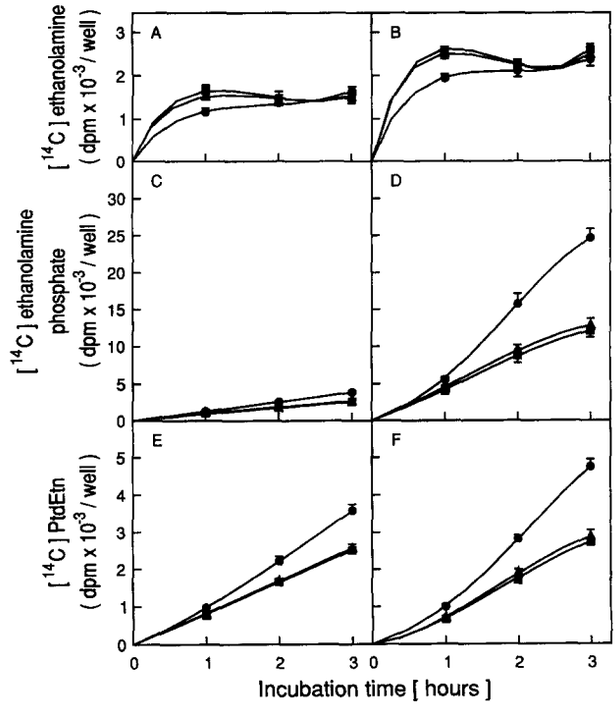


Fig. 1. Effects of DMBA and wortmannin on the uptake and metabolism of [^{14}C]Etn in C3H/10T1/2 fibroblasts. Fibroblasts, grown in 12-well culture dishes up to confluency, were untreated (A,C,E), or were treated for 23 h with 0.5 μg/ml of DMBA (B,D,F). This was followed by incubation (without removing DMBA) of fibroblasts for 1 h in the absence (●) or presence of 50 nM (▲) or 200 nM (■) wortmannin. At this time point, each well received (without removing DMBA/wortmannin) [^{14}C]Etn (50 μM; 991,000 dpm/well), and incubations continued for up to 3 h. Incorporation of exogenous [^{14}C]Etn into the cellular pool of [^{14}C]Etn (A,B), [^{14}C]EtnP (C,D) and [^{14}C]PtdEtn (E,F) were determined as described in section 2. Each point represents the mean ± S.E.M. of eight independent incubations.

the increase in the cellular content of [^{14}C]Cho reached a plateau after 2 h incubation. In both untreated and DMBA-treated fibroblasts, addition of wortmannin slightly enhanced the cellular content of [^{14}C]Cho. In DMBA-treated fibroblasts (Fig. 2D), the rate of [^{14}C]ChoP formation at increasing incubation times was 3.9- to 10.1-fold higher than in untreated fibroblasts (Fig. 2C). Also, 50–200 nM concentrations of wortmannin inhibited the formation of [^{14}C]ChoP both in the untreated (Fig. 2C) and DMBA-treated fibroblasts (Fig. 2D) to similar extents, as it was observed in the case of [^{14}C]EtnP formation. Again, despite the large stimulatory effects of DMBA on [^{14}C]ChoP formation, incorporation of [^{14}C]Cho into PtdCho occurred to similar extents in the untreated (Fig. 2E) and DMBA-treated fibroblasts (Fig. 2F). Furthermore, wortmannin similarly inhibited the formation of [^{14}C]PtdCho in the untreated and DMBA-treated fibroblasts. We should add here that treatment of subconfluent cultures with DMBA resulted in about 1.5- to 2.4-fold stimulation of [^{14}C]ChoP formation over a 3 h incubation period (data not shown).

Previously, a treatment of subconfluent [^{14}C]Etn-labeled fibroblasts with DMBA for 24 h enhanced the cellular level of [^{14}C]EtnP about 2-fold, but DMBA caused no significant increase in the cellular level of [^{14}C]ChoP [1]. We repeated this experiment with confluent ^{14}C-labeled C3H/10T1/2 fibroblasts

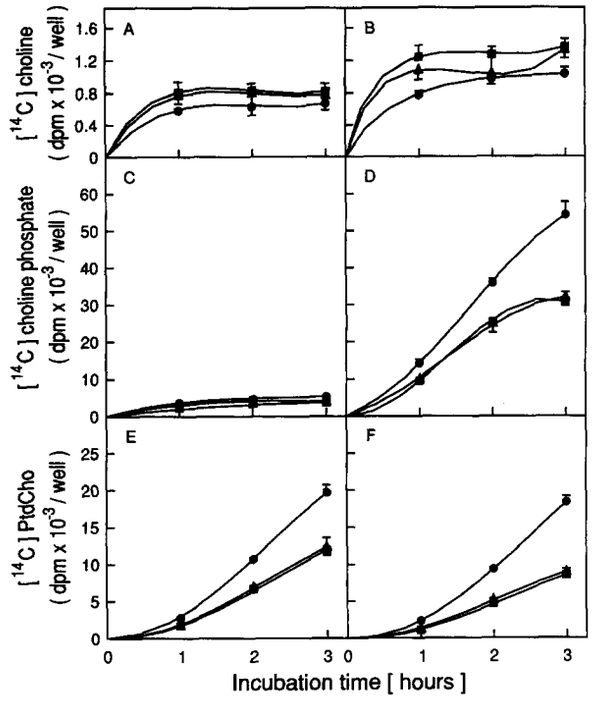


Fig. 2. Effects of DMBA and wortmannin on the uptake and metabolism of [^{14}C]Cho in C3H/10T1/2 fibroblasts. The schedule of treatment of confluent fibroblast cultures with DMBA and wortmannin was the same as described in legend to Fig. 1. Untreated (A,C,E) and DMBA-treated (B,D,F) fibroblasts were incubated in the presence of [^{14}C]Cho (50 μM; 1.28 × 10^6 dpm/well) for up to 3 h. During the labeling period, wortmannin was not present (●), or it was present at 50 nM (▲) or 200 nM (■) concentration. Incorporation of exogenous [^{14}C]Cho into the cellular pool of [^{14}C]Cho (A,B), [^{14}C]ChoP (C,D) and [^{14}C]PtdCho (E,F) were determined as described in section 2. Each point represents the mean ± S.E.M. of eight independent incubations.

and found that treatments with 0.5 μg/ml of DMBA for 24 h enhanced the cellular levels of [^{14}C]EtnP and [^{14}C]ChoP 3.9- and 1.8-fold, respectively (data not shown). Thus, in confluent fibroblasts, increased formation of [^{14}C]EtnP remained the dominant long-term effect of DMBA.

The ability of wortmannin to inhibit phosphorylation of Etn and Cho, both in untreated and DMBA-treated C3H/10T1/2 fibroblasts, would be consistent with a direct effect of this drug on the respective kinase activity. However, in confluent NIH3T3 fibroblasts, wortmannin failed to inhibit phosphorylation of either Etn or Cho (Fig. 3A). On the other hand, a treatment of these fibroblasts with DMBA enhanced the rate of phosphorylation of both [^{14}C]Etn and [^{14}C]Cho about 2.4- to 2.6-fold, and these effects of DMBA were significantly (by 31–33%) inhibited by 200 nM wortmannin (Fig. 3B). Clearly, wortmannin could not directly affect the activity of Etn/Cho kinase.

At the concentrations used here, wortmannin has been reported to be a specific inhibitor of PI3K [7]. Thus, this enzyme may well regulate Etn/Cho kinase activity establishing a direct link between the inhibitory effects of wortmannin on the activity of PI3K and phosphorylation of Etn/Cho. Insulin is a major activator of PI3K (referenced in [8]) and PI3K appears to play a role in the mediation of mitogenic effects of insulin [8]. In accordance with such mechanisms, we also found that in NIH 3T3 fibroblasts, wortmannin suppressed the stimulatory effect of insulin on DNA synthesis (Fig. 4). In these fibroblasts, the stimulatory effect of insulin on DNA synthesis can be enhanced by 1 mM Etn. While the mechanism of the potentiating effect of Etn remains to be determined, the large combined stimulatory effects of insulin plus Etn were also greatly inhibited by wortmannin (Fig. 4). However, using similar conditions, we could not observe an insulin effect on the phosphorylation of either Etn or Cho (data not shown).

4. Discussion

The first major finding of this work was that the chemical carcinogen DMBA stimulated phosphorylation of not only Etn but also that of Cho. After a 3 h labeling period, a preceding DMBA treatment for 24 h enhanced the phosphorylation of [^{14}C]Cho and [^{14}C]Etn about 10.1- and 6.4-fold, respectively. Despite the greater effect of DMBA on the phosphorylation of Cho added to the medium, a longer-term (24 h) treatment of ^{14}C-labeled fibroblasts with DMBA preferentially enhanced the cellular level of [^{14}C]EtnP. Two possible mechanisms may account for this apparent discrepancy in the results. The effect of DMBA on Cho phosphorylation might be more transient compared to the stimulation of Etn phosphorylation. The other possibility is that this kinase, due to the compartmentalization of this system, preferentially used Etn and Cho derived from phospholipid hydrolysis. In this latter case it would mean preferential use of Etn, because DMBA increased the formation of ethanolamine from PtdEtn, but it decreased the formation of choline from PtdCho [6]. We are presently investigating both possibilities. Regardless of the exact mechanisms involved,

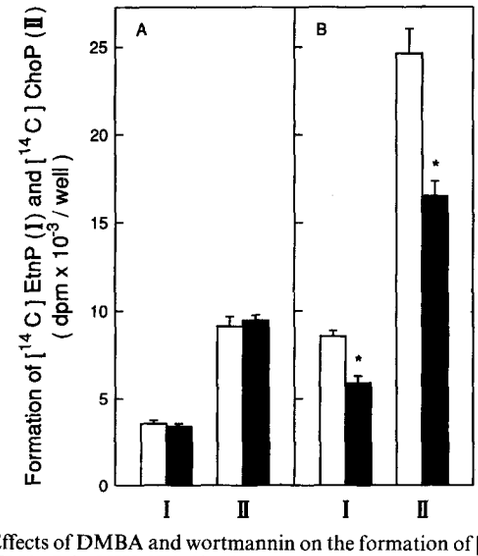


Fig. 3 Effects of DMBA and wortmannin on the formation of [^{14}C]EtnP and [^{14}C]ChoP in NIH 3T3 fibroblasts. Fibroblasts, grown in 12-well culture dishes up to confluency, were untreated (A), or were treated for 23 h with 0.5 μg/ml of DMBA (B). This was followed by incubation (without removing DMBA) of fibroblasts for 1 h in the absence (□) or presence of 200 nM wortmannin (■). At this time point, each well received (without removing DMBA/wortmannin) 50 μM [^{14}C]Etn (802,000 dpm/well) on 50 μM [^{14}C]Cho (1.23 × 10^6 dpm/well) and incubations continued for 2 h. Formation of [^{14}C]EtnP (I) and [^{14}C]ChoP (II) was determined as indicated in section 2. Data represent the mean ± S.E.M. of six independent incubations. *Significantly ($P < 0.01$) different from the respective control value (Student's t-test).

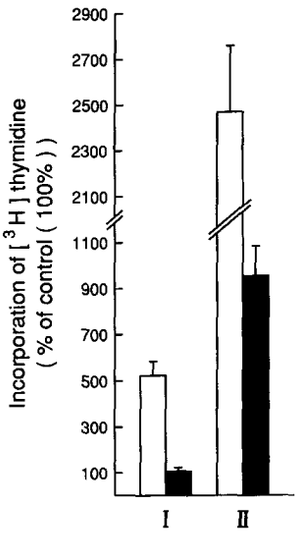


Fig. 4. Inhibition of insulin-induced DNA synthesis by wortmannin in NIH 3T3 fibroblasts. Fibroblasts, treated with 500 nM insulin (I) or 500 nM insulin plus 1 mM Etn (II) were incubated in the presence of [^{3}H]thymidine as described in section 2. During the treatment (16 h) and labeling (1 h) period, 50 nM wortmannin was either absent (□) or present (■). Data are the mean ± S.E.M. of three incubations. The effects of ethanolamine and wortmannin were confirmed in two other experiments each performed in triplicate.

however, it is clear that at least in fibroblasts, a longer exposure to the carcinogen preferentially enhances the cellular level of EtnP, even though the activated kinase phosphorylates both Etn and Cho. This may be relevant to other observations indicating that in several human tumors it is EtnP, and not ChoP, whose tissue level is enhanced [9–15].

The second major finding of this work was that wortmannin, an inhibitor of PI3K [7], also inhibited phosphorylation of both Cho and Etn. The fact that wortmannin specifically inhibited phosphorylation of [^{14}C]Cho and [^{14}C]Etn without reducing the cellular levels of these precursors provide an important proof that this drug inhibited the respective kinase activity, and not the uptake mechanisms. Furthermore, these data also serve as additional evidence that the primary target of DMBA action was the Etn/Cho kinase system. On the other hand, it is important to stress that inhibition of kinase activity by wortmannin did not result in large increases in the cellular levels of Cho and Etn. This suggests that a higher rate of production of ChoP and EtnP by activated kinase is accompanied by stimulated uptake of precursors and vice versa.

Importantly, insulin, a major activator of PI3K [8], failed to enhance phosphorylation of Cho or Etn in NIH 3T3 fibroblasts. A possible interpretation of this result is that the inhibitory effects of wortmannin on the Etn/Cho kinase activity are not mediated by PI3K. Alternatively, two cellular pools of PI3K may exist; an insulin-sensitive pool of PI3K may not regulate, while an insulin-insensitive pool of PI3K may regulate Etn/Cho kinase activity. Further work is needed to clarify this important issue.

The inhibitory effect of wortmannin on the activity of Etn/Cho kinase is unlikely to reflect a direct interaction of wortmannin with this enzyme because in untreated NIH 3T3 fibroblasts this drug failed to inhibit the phosphorylation of Etn and Cho. While these latter data indicate that inhibition of this kinase activity by wortmannin occurs by an indirect mechanism, they also indicate that the mechanism which accounts for the stimulatory effects of DMBA is partially activated in C3H/10T1/2 fibroblasts, but not in NIH 3T3 fibroblasts.

Finally, we should point out that the higher levels of [^{14}C]EtnP and [^{14}C]ChoP in DMBA-treated fibroblasts were not reflected in increased synthesis of the respective phospholipids. Clearly, phosphorylation of ChoP and EtnP is not the rate-limiting step in the synthesis of these phospholipids, an observation which agrees well with observations from several other laboratories (reviewed in refs. 16 and 17). These results indicate that if carcinogen-induced synthesis of EtnP and ChoP has indeed any role in the transformation process, this is not mediated by phospholipid synthesis.

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