Ha-Ras stimulates uptake and phosphorylation of ethanolamine: inhibition by wortmannin

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Abstract Transformation of NIH 3T3 fibroblasts by Ha-Ras resulted in large increases in the phosphorylation of both [^{14}C]ethanolamine (Etn) and [^{14}C]choline (Cho) when these precursors were added to the medium. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), preferentially decreased phosphorylation of externally added Etn in the Ha-Ras transformed, but not in the untransformed, fibroblasts. However, wortmannin had no effect on the phosphorylation of Etn formed endogenously by phorbol ester-stimulated hydrolysis of phosphatidylethanolamine. Data suggest that interaction of mutated Ras with PI3K leads to specific stimulation of Etn uptake, followed by nearly quantitative phosphorylation of Etn by a Ras-activated Cho/Etn kinase.

Key words: Ras; Ethanolamine; Choline; Kinase; NIH 3T3 fibroblasts; Phosphatidylinositol 3-kinase

1. Introduction

Ras genes encode proteins which regulate key signaling pathways involved in cell growth and development [1–5]. One of the many actions of activated Ras is stimulation of expression/activity of choline (Cho) kinase [6]. In view of a recent report [7] demonstrating that choline phosphate (ChoP) is a positive regulator of cell growth, stimulation of Cho kinase activity by activated (oncogenic) Ras may be required for the rapid growth of transformed cells.

We have recently reported [8] that the environmental carcinogens benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene enhanced both the formation of ethanolamine (Etn) from phosphatidylethanolamine (PtdEtn) and subsequent phosphorylation of Etn to Etn phosphate (EtnP). Since the transforming activity of carcinogens may involve activation of Ras [9–12], and because in most cases Cho kinase also phosphorylates Etn, it seemed likely that activated Ras, such as present in Ha-Ras-transformed fibroblasts, will also phosphorylate Etn to a greater extent. The major goal of this work was to examine this possibility.

Phosphatidylinositol 3-kinase (PI3K) has recently been found to be a direct target of Ras [13]. This prompted us to examine the possible role of PI3K in the mediation of Ras effects on Cho/Etn kinase activity. Since wortmannin has been reported to be a specific potent inhibitor of PI3K [14], the possible effects of this drug on the phosphorylation of Etn and Cho in untransformed and Ha-Ras-transformed NIH 3T3 fibroblasts were also determined.

2. Experimental

2.1. Materials

Wortmannin, phorbol 12-myristate 13-acetate (PMA), Etn, Cho and Dowex-50W[H$^+$ form] were purchased from Sigma; [2-^{14}C]ethanolamine (55 mCi/mmol) and [methyl-^{14}C]choline (50 mCi/mmol) were bought from Amersham; tissue culture reagents were from Gibco-BRL.

2.2. Cell culture

NIH 3T3 clone 7 fibroblasts transfected with Harvey-murine-sarcoma virus [15] as well as the parent cell line were obtained from Dr. Douglas R. Lowy (National Cancer Institute, Bethesda, MD, USA). Cells were cultured continuously in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum, penicillin/streptomycin (50 units/ml and 50 μg/ml, respectively) 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 incubated in the presence of wortmannin (50–200 nM) for 1 h. This was followed (with no washing step being included) by incubations of fibroblasts for 30–150 min in the presence of 50 μM [^{14}C]Etn (955,000 dpm/well) or 50 μM [^{14}C]Cho (1.21×10^6 dpm/well). At the end of incubations, the incubation medium was aspirated, then fibroblasts were gently, but rapidly (within 20 s), washed with 5 ml medium followed by the addition of ice-cold methanol (2 ml) to the wells. Then, cells were scraped into methanol, and methanol extracts were rapidly transferred to 2 ml of chloroform. The water-soluble metabolites of [^{14}C]Etn and [^{14}C]Cho were fractionated on Dowex-50W[H$^+$]-packed columns (Bio-Rad Econo columns, 1 ml bed volume) as described by Cook and Wakelam [16], with the following modifications. The initial flow-through (4.5 ml) along with a following 3.5- or 5-ml water wash contained glycerophosphoethanolamine or glycerophosphocholine, respectively. EtnP and ChoP were eluted by 15 and 20 ml water, respectively. Finally, Etn and Cho were eluted by 12 and 20 ml of 1 M HCl, respectively. The metabolites of [^{14}C]Etn and [^{14}C]Cho were identified and phospholipids were separated as indicated previously [17].

2.4. Determination of Etn and EtnP formation by phospholipase-mediated hydrolysis of PtdEtn in fibroblasts

Ha-Ras-transformed and untransformed fibroblasts were grown as above to near confluency in the presence of [^{14}C]Etn (1 μCi/well; 48 h incubation). Fibroblasts were washed and then incubated in fresh medium for 4 h to achieve acceptable background levels of [^{14}C]Etn (210–250 dpm/well) and [^{14}C]EtnP (1030–1140 dpm/well); wortmannin was added for the last 1 h of the 4-h incubation period. At this point, only PtdEtn was significantly labeled (305,000–322,000 dpm/well). This step was followed by treatment of fibroblasts (in the continuous presence of wortmannin, when appropriate) for 2 h with 100 nM PMA in the presence of 50 μM or 1 mM unlabeled Etn. Formation of water-soluble products of [^{14}C]PtdEtn hydrolysis was determined as above, except that the incubation medium was not removed before the addition of ice-cold methanol.

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Abbreviations: Ha-Ras, Harvey-Ras; Etn, ethanolamine; EtnP, ethanolamine phosphate; Cho, choline; ChoP, choline phosphate; PI3K, phosphatidylinositol 3-kinase; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; PMA, phorbol 12-myristate 13-acetate.

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3. Results and discussion

In different species, Etn has been shown to exist in the circulation at concentrations ranging from 5 to 50 μM (referenced in [18]). Therefore, to ensure that these results will be relevant to physiological conditions, in most experiments the concentration of externally added [^{14}C]Etn was 50 μM. When confluent untransformed (Fig. 1A) and Ras-transformed (Fig. 1B) fibroblasts were incubated in the presence of [^{14}C]Etn, the increase in the cellular content of [^{14}C]Etn in both cases reached a plateau after 120–150 min incubation. However, at each time point examined, Ha-Ras transformed cells accumulated somewhat more [^{14}C]Etn consistent with stimulation of the uptake mechanism. At 50 and 200 nM concentrations wortmannin slightly enhanced the cellular content of [^{14}C]Etn in both cell lines. At each time interval examined (30–150 min), the rate of [^{14}C]EtnP formation was 13- to 16-fold greater in the Ras-transformed cells (Fig. 1D) than in the untransformed cells (Fig. 1C). While in the control cells wortmannin had no effect on the formation of [^{14}C]EtnP, in the Ras-transformed cells 50 and 200 nM concentrations of wortmannin decreased phosphorylation of Etn by 16 to 37% at various time points. At 200 nM concentration wortmannin was slightly more effective than at

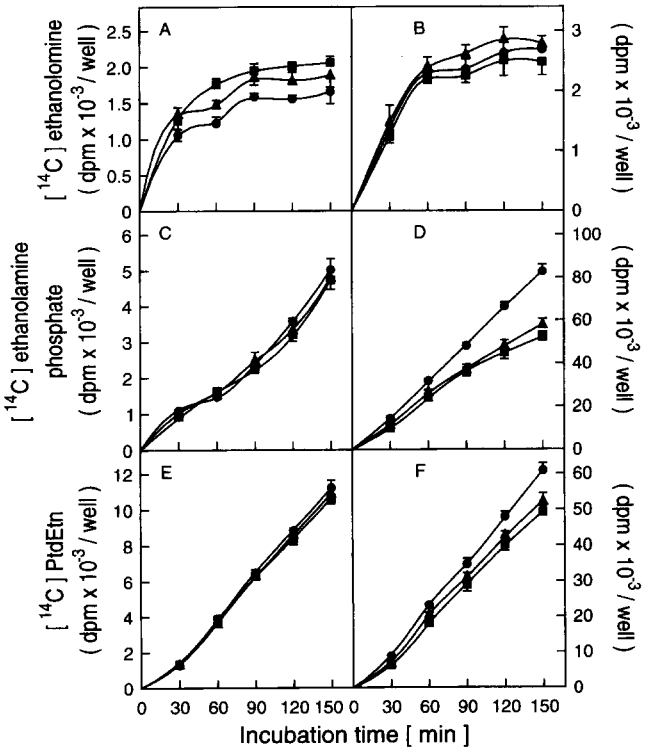

Fig. 1. Uptake and metabolism of [^{14}C]Etn in untransformed and Ha-Ras-transformed NIH 3T3 fibroblasts. Untransformed (A,C,E) and Ras-transformed (B,D,F) fibroblasts, grown in 12-well culture dishes up to confluency, were untreated (●), or were treated with 50 nM (▲) or 200 nM (■) wortmannin for 1 h. At this time point, each well received (without removing wortmannin) [^{14}C]Etn, as described in section 2, and incubations continued for up to 150 min. 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) was determined as described in section 2. Each point represents the mean ± S.E.M. of six independent incubations. At each time point examined, the inhibitory effects of 50–200 nM wortmannin were statistically significant ($P < 0.05$–0.01) by Student's t-test. Similar results were obtained in another experiment performed in triplicate.

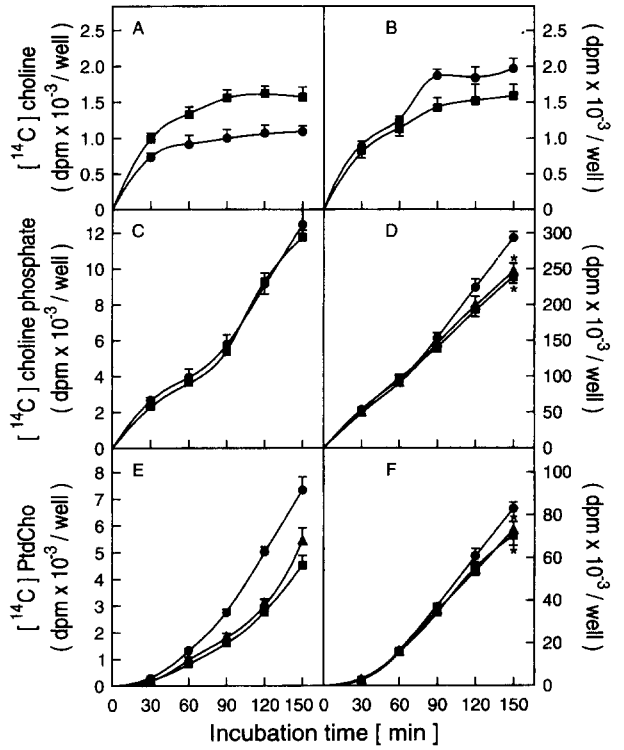

Fig. 2. Uptake and metabolism of [^{14}C]Cho in untransformed and Ha-Ras-transformed NIH 3T3 fibroblasts. Untransformed (A,C,E) and Ras-transformed (B,D,F) fibroblasts, grown in 12-well culture dishes up to confluency, were untreated (●), or were treated with 50 nM (▲) or 200 nM (■) wortmannin for 1 h. At this time point, each well received (without removing wortmannin) [^{14}C]Cho, as described in section 2, and incubations continued for up to 150 min. 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) was determined as described in section 2. Each point represents the mean ± S.E.M. of eight independent incubations. In some cases, the effect of 50 nM wortmannin is not shown; this is to avoid confusion because 50 nM and 200 nM concentrations caused practically the same effect. *P ($P < 0.05$) different from the respective control value (Student's t-test).

50 nM concentration, while at both concentrations its inhibitory effects did not appear to significantly depend on the length of incubation time.

Stimulation of [^{14}C]EtnP formation in the Ras-transformed cells was reflected in a much higher rate of [^{14}C]PtdEtn formation (Fig. 1F) compared to untransformed cells (Fig. 1E). Similarly, wortmannin decreased ^{14}C-labeling of PtdEtn only in the Ras-transformed cells (Fig. 1F), but not in the untransformed cells (Fig. 1E).

Accumulation of [^{14}C]Cho by confluent cultures of untransformed (Fig. 2A) and Ras-transformed cells (Fig. 2B) similarly reached a plateau after 120 min incubation. Ha-Ras-transformed cells again accumulated more [^{14}C]Cho compared to untransformed cells. At 200 nM concentration wortmannin slightly enhanced (maximum 1.5-fold) or slightly decreased (maximum by 19%) the [^{14}C]Cho content of untransformed and Ras-transformed fibroblasts, respectively.

The rate of [^{14}C]ChoP formation was 20- to 24-fold greater in the Ras-transformed cells (Fig. 2D) compared to the untransformed cells (Fig. 2C). Even 200 nM wortmannin failed to modify the formation of [^{14}C]ChoP in the untransformed

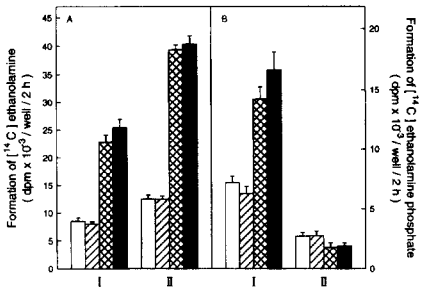

Fig. 3. Effects of wortmannin on PMA-induced formation of [^{14}C]Etn and [^{14}C]EtnP from [^{14}C]PtdEtn in Ha-Ras-transformed fibroblasts. Ha-Ras-transformed fibroblasts were prelabeled with [^{14}C]Etn and then treated with wortmannin as described in section 2. Prelabeled fibroblasts were incubated for 2 h in the absence (□) or presence of 200 nM wortmannin (▨), 100 nM PMA (▩), or wortmannin plus PMA (■); the incubation medium also contained 50 μM (I) or 1 mM (II) unlabeled Etn. [^{14}C]Etn and [^{14}C]EtnP were determined as described in section 2. Data are the mean ± S.E. of six independent incubations.

cells. Wortmannin also failed to decrease [^{14}C]ChoP formation in the Ras-transformed cells during the first 90 min of treatment, but after treatments for 150 min it decreased [^{14}C]ChoP formation by 16 to 19% (Fig. 2D).

Surprisingly, in untransformed fibroblasts wortmannin even at the lower (50 nM) concentration significantly inhibited the incorporation of [^{14}C]choline into PtdCho (Fig. 2E). Since wortmannin failed to inhibit [^{14}C]ChoP formation in these cells, inhibition of PtdCho synthesis should have occurred either at the level of cytidylyltransferase or at the final synthetic step. The effect of wortmannin on PtdCho synthesis was clearly different from that on EtnP formation and was not pursued further here. In the Ras-transformed cells (Fig. 2F) the rate of PtdCho labeling/synthesis was about 10 times greater than in the control cells (Fig. 2E). In the transformed cells, wortmannin had no significant effect on PtdCho synthesis up to 120 min, but it slightly (by 12–15%), although significantly, decreased the incorporation of [^{14}C]Cho into PtdCho after 150 min incubation (Fig. 2F). It should be added here that extension of incubation period up to 6 h failed to increase the inhibitory effect of wortmannin on [^{14}C]ChoP formation (data not shown).

Etn and Cho are presumably phosphorylated by the same kinase activity [19], while transport of these precursors across the cell membrane involves different mechanisms [18]. Therefore, it was possible that wortmannin actually inhibited the uptake of [^{14}C]Etn and this process was reflected in decreased formation of [^{14}C]EtnP. However, due to the presence of an extremely active kinase activity in the transformed cells and to the absence of a specific kinase inhibitor, it is virtually impossible to directly determine possible inhibition of [^{14}C]Etn uptake by wortmannin. However, it is possible to endogenously generate a large intracellular pool of [^{14}C]Etn by PMA-induced, phospholipase D-mediated, hydrolysis of prelabeled PtdEtn. We have previously reported that transformation of NIH 3T3 fibroblasts by Ha-Ras actually enhanced the effect of PMA on PtdEtn hydrolysis [20]. As shown in Fig. 3, at 50 μM concentration of Etn in the medium PMA-treated [^{14}C]Etn-prelabeled Ha-Ras-transformed fibroblasts generated significant amounts of both [^{14}C]Etn and [^{14}C]EtnP, while at 1 mM Etn PMA induced only [^{14}C]Etn formation. This indicates the 50 μM Etn only partially, while 1 mM Etn fully inhibited phosphorylation of [^{14}C]Etn formed by PMA-induced hydrolysis of [^{14}C]PtdEtn. Importantly, wortmannin (200 nM) inhibited neither the phosphorylation of [^{14}C]Etn (at 50 μM Etn) nor the formation of [^{14}C]Etn (at 1 mM Etn). These data clearly indicate that wortmannin does not directly inhibit phosphorylation of Etn, and it is also without effect on phospholipase D activity.

In summary, these results demonstrate for the first time that activated Ras greatly enhances the Etn-phosphorylating capacity of cells, along with the stimulation of ChoP formation. This is consistent with Etn and Cho being phosphorylated by the same kinase activity [19]. Clearly, Ha-Ras also stimulates the uptake of these precursors. The results indicating that wortmannin inhibited only the phosphorylation of externally added Etn, but not of Etn formed endogenously from PtdEtn, strongly suggest that PI3K, activated by mutated Ras [13], positively regulates Etn transport. Many human cancers are characterized by increased levels of EtnP, but not ChoP [21–27]. This suggests that aggressive growth of certain cancers requires high levels of EtnP, which is achieved through the stimulation of both uptake and phosphorylation of Etn. The present findings, implicating both activated Ras and PI3K in the regulation of Etn metabolism, represent an important step toward determination of the possible role of Etn/EtnP in human cancers.

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