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Signal transduction in EJ-H-*ras*-transformed cells: de novo synthesis of diacylglycerol and subversion of agonist-stimulated inositol lipid metabolism

Vincenzo P. Chiarugi, Lucia Magnelli, Franca Pasquali, Gabriele Basi and Marco Ruggiero

Laboratory of Molecular Biology, Institute of General Pathology, University of Firenze, Viale Morgagni 50, 50134 Firenze, Italy

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We examined the level of 1,2-diacylglycerol and inositol phosphates in normal and EJ-H-*ras*-transformed BALB/3T3 fibroblasts by prelabelling the cells with [^{3}H]glycerol, [^{3}H]inositol, [^{14}C]glucose, [^{14}C]arachidonic acid, and [^{14}C]palmitic acid. Steady-state level of diacylglycerol was increased in *ras*-transformed cells prelabelled with radioactive glycerol, glucose and palmitic acid. Steady-state level of inositol phosphates, however, was the same in control and transformed cells. Diacylglycerol labelling by [^{14}C]arachidonic acid was the same in control and transformed cells. Insulin dramatically increased diacylglycerol labelling by [^{14}C]glucose in normal cells, wheras it did not affect *ras*-transformed fibroblasts. Neurotransmitter-induced inositol lipid turnover was greatly enhanced in *ras*-transformed cells; conversely, platelet-derived growth factor and thrombin-stimulated normal cells to a greater extent than transformed fibroblasts. Taken together these results suggest that *ras* transformation may induce multifarious effects on signal transduction: it may cause de novo synthesis of diacylglycerol and subversion of neurotransmitter and growth factor receptor coupling to inositol lipid metabolism.

Oncogene, *ras*; Inositol lipid; Diacylglycerol; Signal transduction

1. INTRODUCTION

Since our first observation of altered inositol lipid metabolism in *ras*-transformed cells [1], several reports have been published on this controversial issue [2–19]. There seems to be general agreement that *ras*-transformed cells have increased steady-state level of 1,2-diacylglycerol (DAG), although discrepancies arise concerning the source of this DAG. It has been proposed that DAG might derive from constitutive hydrolysis of inositol lipids [3,12–15], or other phospholipids [6,7,11]; however, these hypotheses have been recently questioned [18,20], and no alternative explanation for this phenomenon is currently available. Another point of controversy concerns the responsiveness of *ras*-transformed cells to a number of agents that stimulate inositol lipid metabolism [1,2,5,9,10,16–18]. Increased or decreased responses have been reported and no conclusive evidence has been reached so far.

In this study we approached the problem from a different perspective. Based on the old concept that transformed cells show increased glucose transport and glycolysis, we investigated whether DAG was neosynthesized along this pathway. Since it has been demonstrated that insulin induces de novo synthesis of DAG [21,22], we studied the effect of insulin on DAG production in control and *ras*-transformed BALB/3T3 fibroblasts. Finally, we reinvestigated the effect of

Correspondence address: M. Ruggiero, LCMB, National Cancer Institute, Bldg. 37, room 1E24, Bethesda, MD 20892, USA

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neurotransmitters and growth factors on inositol lipid metabolism in *ras*-transformed cells. Our results raise the possibility that *ras* transformation may induce multifaceted alterations in signal transduction, influencing DAG neosynthesis and the coupling between different receptors and inositol lipid turnover.

2. MATERIALS AND METHODS

myo-[2-^{3}H]Inositol (10–20 Ci·mmol^{-1}), [2-^{3}H]glycerol (1 Ci·mmol^{-1}), D-[U-^{14}C]glucose (10–15 mCi·mmol^{-1}), [U-^{14}C]arachidonic acid (300–1200 mCi·mmol^{-1}), and [U-^{14}C]palmitic acid (>500 mCi·mmol^{-1}) were from NEN (Dupont). Thin-layer chromatography (TLC) plates (LD6D) were from Whatman. Lipid standards were from Sigma. Platelet-derived growth factor (PDGF) was from Collaborative Research. Thrombin, phenylephrine, carbamylcholine, atropine, prazosin, and insulin were from Sigma. All other reagents were analytical grade or the best commercially available.

Normal BALB/3T3 and EJ-T24-H-*ras*-transformed fibroblasts were kindly provided by Dr Bignami, Istituto Superiore di Sanità, Rome, Italy. This *ras* cloned cell line contains about 15 copies of the integrated H-*ras* oncogene. Cell cultures were normally grown in 50-mm dishes in DMEM containing fetal calf serum. It has been observed that DAG and phosphoinositide levels in normal and *ras*-transformed cells are affected by conditions of cell density [3]. This observation may reflect the fact that normal cells at high density enter a quiescent state, whereas transformed cells continue to proliferate [18]. Therefore, at the time of each experiment, both normal and transformed cell lines were assayed as semiconfluent monolayers containing the same number of cells. For experiments involving measurement of steady-state levels of DAG and inositol phosphates (InsPs), cells were prelabelled to equilibrium with different radioisotopes following the method described in Lacal et al. [11]. After radiolabelling, the cultures were washed and maintained for only 4 h in serum-free medium, after which the medium was removed and cells incubated for 1 h in serum-free medium. This procedure of short serum starvation seems to provide the best experimental conditions for comparing second messenger levels in normal and *ras*-transformed fibroblasts [11]. For experiments involving measurement of incorporation of different precursors into DAG and phosphatidic acid (PA), we followed the procedure described by Macara [23] and Peter-Riesch [22]. Also in these experiments, cultures were serum starved for 4 h prior to the measurements. For experiments designed to measure inositol lipid metabolism in response to agonists, cells were prelabelled to equilibrium with [^{3}H]inositol. Incubation with different agents was performed at 37°C for 10 min in the presence of LiCl (10 mM). Neurotransmitter receptor antagonists, when present, were added 5 min before the respective agonist. InsPs and PA were extracted and separated according to the procedure described in Lapetina et al. [24] as modified by Chiarugi et al. [25]. DAG was separated by TLC as described in Lacal et al. [11]. All data shown were normalized for the radioactivity content in total phospholipids [3,11,25].

Table 1

Steady-state level of diacylglycerol in control and *ras*-transformed fibroblasts prelabelled with different precursors

Radioactive precursor	BALB/3T3	EJ-H-*ras*
[^{3}H]Glycerol	1527 ± 156	4987 ± 189
[^{14}C]Glucose	3455 ± 107	9888 ± 1007
[^{14}C]Palmitic acid	4567 ± 343	10345 ± 876
[^{14}C]Arachidonic acid	2145 ± 402	2986 ± 78

Normal and *ras*-transformed fibroblasts were labelled to equilibrium with 0.5 μCi/ml of [^{14}C]glucose, [^{14}C]palmitic acid, [^{14}C]arachidonic acid, or 2 μCi/ml of [^{3}H]glycerol for 48 h. Steady-state level of diacylglycerol was measured following the procedure described in Lacal et al. [11]. Data are shown as cpm recovered in diacylglycerol (means ± SE, n = 3)

3. RESULTS

Table 1 shows that EJ-H-*ras*-transformed BALB/3T3 fibroblasts had increased steady-state level of DAG when prelabelled to equilibrium with [^{3}H]glycerol, [^{14}C]glucose, and [^{14}C]palmitic acid. Cells prelabelled with [^{14}C]arachidonic acid, however, did not show significant increase of DAG labelling. This phenomenon was dramatically evident under non-equilibrium conditions (fig.1). Labelled DAG and PA were rapidly formed from precursors such as [^{14}C]glucose, [^{3}H]glycerol and [^{14}C]palmitic acid in *ras*-transformed cells; conversely, [^{14}C]arachidonic acid-labelled DAG and PA were formed to the same extent in control and transformed fibroblasts. These results suggest that DAG may be neosynthesized from the glycolytic intermediate dihydroxyacetone phosphate and from glycerol 3-phosphate after step-wise acylation to lysoPA and PA [22]. This hypothesis is supported by the observation indicating that DAG and PA were preferentially labelled by [^{14}C]palmitic acid rather than [^{14}C]arachidonic acid in *ras*-transformants (table 1 and fig.1). As elegantly demonstrated by Peter-Riesch et al. [22], palmitic acid-rich DAG is formed through a neosynthetic pathway, whereas arachidonic acid-rich DAG derives from phospholipase C-mediated hydrolysis of phospholipids.

Since it has been reported that insulin increases

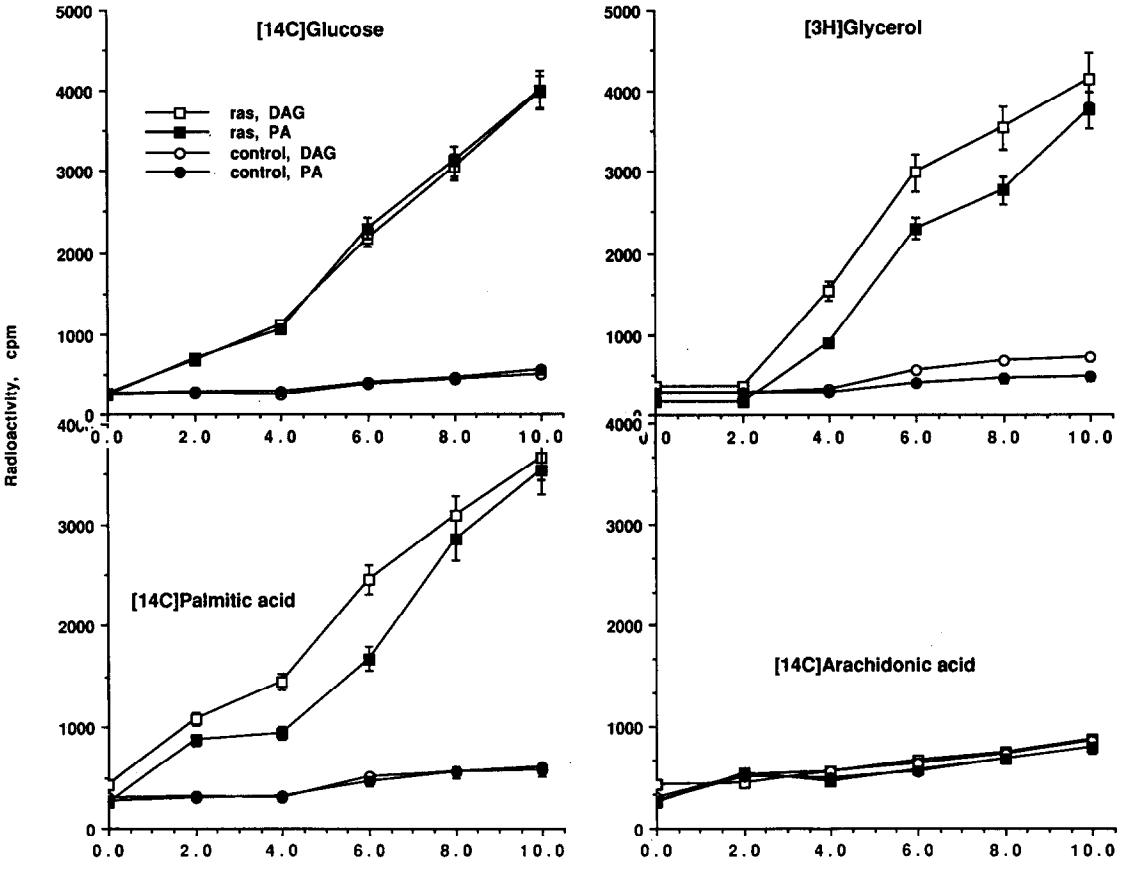

Fig.1. Incorporation of different labelled precursors into diacylglycerol and phosphatidic acid in control and *ras*-transformed BALB/3T3 fibroblasts. Semiconfluent monolayers of normal and EJ-H-*ras*-transformed BALB/3T3 cells were incubated with 0.5 μCi/ml of [^{14}C]glucose, [^{14}C]palmitic acid, [^{14}C]arachidonic acid, or 2 μCi/ml of [^{3}H]glycerol. After addition of each radiolabelled compound, the cells were extracted at different time intervals (minutes, as indicated on the *X* axis). Time 0 indicates when the radiolabelled precursor was added. Extraction and separation of diacylglycerol and phosphatidic acid were performed as described. Data are presented as means ± SE (n = 3).

DAG by stimulating de novo PA synthesis [21,22], we studied the effect of insulin on the rapid kinetics of DAG labelling from [^{14}C]glucose in normal cells and *ras* transformants (fig.2). It is clear that insulin did not modify the incorporation of glucose-derived ^{14}C into DAG in *ras* transformants, whereas it dramatically increased DAG neosynthesis in control fibroblasts.

We then investigated the responsiveness of *ras* transformants to different receptor agonists in terms of InsPs formation. Table 2s hows that *ras*-transformed cells did not exhibit significant changes of the basal level of InsPs when assayed in the presence of LiCl. These results are in agreement with previous reports demonstrating an increase of DAG without a corresponding elevation of InsPs [6,7,11]. However, *ras* transformants showed significantly higher responses to the muscarinic M_1 receptor agonist carbamylcholine, and to the α_1 receptor agonist phenylephrine. These responses were inhibited by the specific receptor antagonists, atropine and prazosin, respectively (table 2). PDGF and thrombin, on the other hand, efficiently stimulated InsPs formation in control BALB/3T3, but failed to induce significant inositol lipid turnover in *ras* transformants.

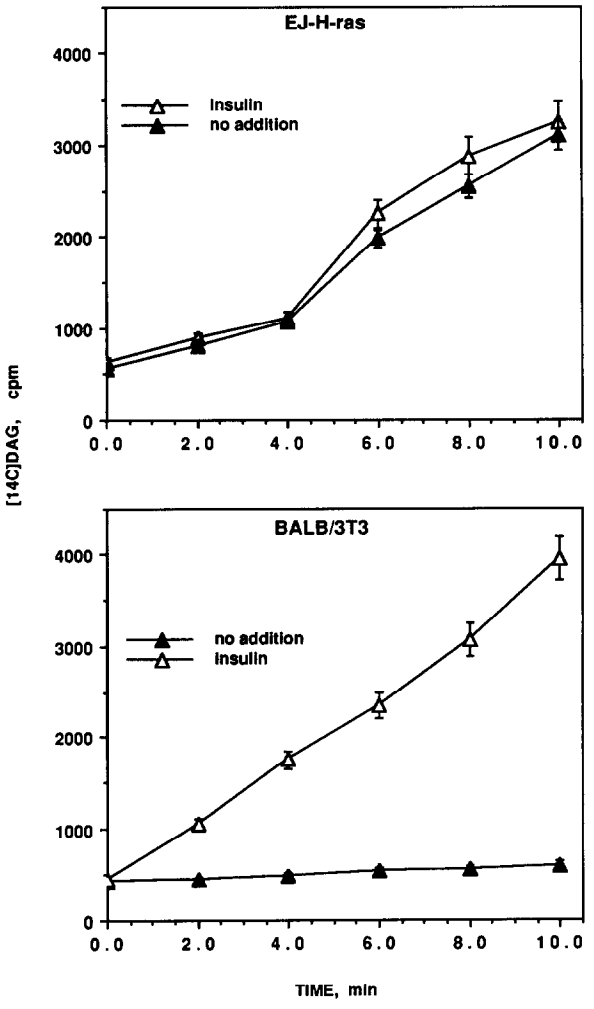

Fig.2. Effect of insulin on the acute transfer of ^{14}C from D-[U-^{14}C]glucose to diacylglycerol in normal and *ras*-transformed fibroblasts. Experimental conditions were as described in the legend to fig.1. Insulin (1 μM) was added together with [^{14}C]glucose at the point indicated as time 0. Results are the means of three experiments ± SE (n = 3).

Table 2

Inositol phosphate formation in response to receptor agonists in normal and *ras*-transformed fibroblasts

Treatment	BALB/3T3	EJ-H-*ras*
None	970 ± 17	1011 ± 215
Carbamylcholine	1207 ± 480	4690 ± 180
Atropine + carbamylcholine	1050 ± 23	1757 ± 150
Phenylephrine	1075 ± 134	3584 ± 98
Prazosin + phenylephrine	1090 ± 154	1685 ± 78
Thrombin	2986 ± 187	1344 ± 41
PDGF	3160 ± 191	1439 ± 43

Cells prelabelled to equilibrium with [^3H]inositol, were incubated with each agonist for 10 min at 37°C in the presence of LiCl (10 mM). Inositol phosphates were extracted and separated as described [24,25]. Data are presented as cpm associated with the total inositol phosphate fraction (means ± SE, n = 3). Carbamylcholine, 10 mM; atropine, 1 mM; phenylephrine, 100 μM; prazosin, 100 μM; thrombin, 10 U/ml; PDGF, 10 ng/ml

4. DISCUSSION

The data presented in this study suggest that *ras*-transformed cells show complex alterations of signal transduction. In particular, we observed that *ras* transformants had elevated steady-state level of neosynthesized DAG, and altered coupling of growth factor and neurotransmitter receptors to inositol lipid metabolism.

It is well accepted that DAG can be produced in response to different agonists through pathways other than inositol lipid hydrolysis [26,27]. Insulin in particular seems to cause de novo synthesis of DAG from the glycolytic pathway in different cell types [21,22,28]. Insulin and p21^{ras} appear to be functionally related: it has been proposed that some of the effects of insulin might be mediated by p21^{ras} [29,30], and it has been demonstrated that efficient DNA synthesis by oncogenic p21$^{H \triangleq ras}$ only occurs in the presence of insulin [31]. Therefore, de novo synthesis of DAG along the glycolytic pathway might represent the point of convergence of insulin and p21^{ras} action. Consistent with this idea is the observation that insulin can increase DAG neosynthesis in normal cells but not in *ras* transformants (fig.2), as if *ras* transformants had maximally activated insulin-like mechanism(s) for DAG formation. Concerning the molecular forms of neosynthesized DAG, at the moment it is impossible to demonstrate whether it is *sn*-1,2DAG or *sn*-2,3DAG. However, since insulin action and *ras* transformation have profound effects on protein kinase C [6,7,13,19,21,32], it is possible that at least some fraction of this neosynthesized DAG is the *sn*-1,2DAG. In our opinion, it is a racemic mixture of the two forms; however, given the significant increase of DAG in *ras* transformants, even if only 50% of this is the *sn*-1,2DAG form, it could be sufficient to explain the effects on protein kinase C.

Since DAG has been reported to be elevated in cell lines transformed by a number of membrane-associated oncogene products [4,7,33], it will be interesting to investigate whether DAG neosynthesis is a phenomenon peculiar to *ras*, or rather common to other oncogenes. Given the evidence that *ras* neutralizing antibody Y13-259 inhibits not only *ras* transformation but blocks transformation by *src*, *fms*, and *fes*, and the mitogenic effects of serum, PDGF, EGF and phorbol esters [34–36], it is also proposable that $p21^{ras}$ might act as multiple coupler between receptors, membrane-associated oncogene products and downstream mitogenic signalling pathways. However, the picture is becoming increasingly intricate since it has been demonstrated that *ras* is able to generate distinct signals dependent and independent of functional protein kinase C [31].

Concerning the effect of *ras* transformation on agonist-induced inositol lipid metabolism, we observed that neurotransmitter-induced responses were amplified, whereas growth factor-induced effects were inhibited (table 2). Altered level of expression of different receptors might be responsible for this observation. It has been demonstrated that fibroblasts transformed by *ras* and other oncogenes show elevated level of bradykinin receptors and increased responsiveness to the peptide hormone [9,10,37]. However, altered inositol lipid turnover in response to carbamylcholine, bombesin, and PDGF in *ras*-transformed cells is not associated with a change in receptor number [2,5,9]. An alternative explanation for these results might be in the different coupling of growth factor and neurotransmitter receptors to phospholipase C. While neurotransmitter receptors are generally coupled via G-proteins [38], recent data suggest that growth factor receptors directly phosphorylate and activate the enzyme [39]. Therefore $p21^{ras}$ might substitute for G-proteins coupling neurotransmitter receptors to inositol lipid metabolism, whereas persistent activation of protein kinase C elicited by elevated DAG might impair growth factor-dependent signalling. Further studies are required to determine the importance of these alterations of signal transduction in malignant transformation.

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