BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 739–745 (1996)
ARTICLE NO. 1874

Inhibitors of Ras Farnesylation Revert the Increased Resistance to Oxidative Stress in K-*ras* Transformed NIH 3T3 Cells

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Received October 23, 1996

Tumor resistance to oxidative stress prevents the efficacy of cancer therapy based upon a free radical-mediated mechanism. *K-ras* transformed NIH 3T3 cells (E32-4-2) showed, under oxidative stress, reactive oxygen species (ROS) levels 10-fold lower and lipid peroxide levels 56% lower, compared to their nontransformed counterpart. Since $p21^{ras}$ activity depends upon farnesylation, we tested the effect of the inhibitors of farnesylation lovastatin and (α-hydroxyfarnesyl) phosphonic acid on susceptibility to oxidative stress in these cells. Preincubation of cells for 24 h with 10 μM lovastatin resulted in a 10-fold increase of ROS levels and a 50% increase of lipid peroxide levels measured under pro-oxidant conditions. Similarly, preincubation of cells with 100 μM (α-hydroxyfarnesyl) phosphonic acid for 24 h enhanced stress-induced levels of either ROS (7.5-fold) or lipid peroxides (33%). The effect of lovastatin and (α-hydroxyfarnesyl) phosphonic acid is specifically due to their ability to inhibit $p21^{ras}$ activity. In fact, inhibition of $p21^{ras}$ by transfecting E32-4-2 cells with the transdominant negative mutant of H-*ras* (L61,S186) led, analogously to lovastatin or (α-hydroxyfarnesyl) phosphonic acid treatment, to a strong increase of stress-induced ROS levels. These results suggest that farnesylation inhibitors could be used as an adjuvant therapy to improve the tumoricidal effect of cancer treatment based upon free-radical production in *ras*-dependent tumors. © 1996 Academic Press

Tumor tissues as well as transformed cells in culture are usually more resistant to oxidative stress compared to their nontransformed counterpart (1,2,3). This characteristic can interfere with the efficacy of cancer therapy based upon free radical production as radiotherapy or chemotherapy with compounds like doxorubicin or daunorubicin. Therefore there is a growing interest in understanding the mechanisms involved in the enhanced resistance to oxidative stress in tumors as well as in the development of new therapeutic strategies directed to enhance susceptibility to free radical-mediated cell damage in tumor cells.

Ras proteins (H-Ras, K-Ras, N-Ras) are members of the small GTP binding protein family involved in cell growth and differentiation (4). An increased expression of normal or mutated Ras proteins is present in large number of tumors with varying frequency (5) with the highest incidence in pancreas adenocarcinoma (90%), colon and lung tumors (50%), myeloid leukemia and anaplastic and follicular thyroid carcinomas (30%). In previous studies the increase in *ras* gene expression has been related with augmented radioresistance in NIH 3T3 cells (6); at the same time, a strict correlation between *ras* mRNA and $p21^{ras}$ levels and the degree of cell

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Abbreviations: ROS, reactive oxygen species; HMG-CoA reductase, 3-hydroxy 3-methylglutaryl coenzyme A reductase; FPTase, farnesyl protein transferase; TBARS, thiobarbituric acid reactive substances; DCHF-DA, 5,6-carboxy-2′,7′-dichlorofluorescein diacetate; MDA, malondialdehyde.

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radioresistance or doxorubicin and H_2O_2 sensibility was observed in human osteosarcoma subclones that differ in EJ *ras* expression (7,8).

Ras proteins are associated with the plasma membrane and this step is essential for their biological functions. Membrane association is allowed by a series of post-translational modifications of p21^{ras}; the first and obligatory one for its transforming activity consists in the covalent binding of a polyisoprenoid, a farnesyl group, to the cysteine residue located in COOH-terminus tetrapeptide, in a reaction catalyzed by farnesyl protein transferase (FPTase) (9). The early and rate-limiting step of the polyisoprenoid pathway is the conversion of 3-hydroxy 3-methylglutaryl Coenzyme A (HMG-CoA) to mevalonate, catalyzed by HMG-CoA reductase that is down-regulated by v-K-*ras* oncogene (10). Recently new agents that interfere with Ras function by inhibiting post-translational processing of p21^{ras} have been developed (11,12). In this paper we investigate the effect of p21^{ras} activity blockade by inhibitors of farnesylation on reactive oxygen species (ROS) and lipid peroxide levels produced under oxidative stress in K-*ras* transformed NIH 3T3 fibroblasts. On this purpose we used either lovastatin, an HMG-CoA reductase competitive inhibitor, or (α-hydroxyfarnesyl) phosphonic acid, an FPTase inhibitor. The effect of the transdominant negative mutant of H-*ras* (L61, S186) on ROS production in K-*ras* transformed cells was also tested in order to verify the direct involvement of oncogenic *ras* in the response to oxidative stress in these cells.

MATERIALS AND METHODS

Cell lines and cell culture conditions. NIH 3T3 cells are nontransformed murine embryo fibroblasts; their K-*ras* transformed counterpart (E32-4-2 cells), derived from NIH 3T3 cells upon transfection with K-*ras,* were kindly provided by Dr. A. Eva (NCI, Laboratory of Cellular and Molecular Biology, Bethesda, MD) (13). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutammine, 50 μg/ml streptomycin and 50 IU/ml penicillin under a humidified atmosphere composed of 95% air and 5% CO_2 at 37°C.

Inhibition of Ras processing was carried out by incubating cells with 10 μM lovastatin (kindly provided by Dr. Alberts of Merck Sharp and Dohme Co.) or 100 μM (α-hydroxyfarnesyl) phosphonic acid (Calbiochem) at 37°C for 24 h in fresh medium, as indicated.

Fluorescent measurement of intracellular ROS. Cells were trypsinized, collected in polystyrene tubes and washed 3 times with 137 mM NaCl, 3.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.5 (PBS). 1×10^6 cells were suspended in 2 ml of PBS and incubated with 10 μM of 5,6 -carboxy -2′,7′ -dichlorofluorescein diacetate (DCHF-DA; #C-2938, Molecular Probes, Eugene, OR), for 1 h at 37°C and kept in DCHF-DA continuously thereafter. Oxidative stress was induced on half cells by incubation at 37°C with 0.1mM $FeSO_4$ and 0.2 mM sodium ascorbate; 200 μl aliquotes per point of either stressed or unstressed cells were analyzed at the times specified by Fluorescence Activated Cell Sorting (FACS) with excitation and emission setting of 495 and 525 nm, respectively.

Lipid peroxidation measurements. Cells were grown to semiconfluence in 100 mm culture dishes; the cell monolayers were then trypsinized, harvested in polystyrene tube and washed 3 times with PBS. To measure susceptibility to lipid peroxidation under oxidative stress conditions, cell pellets were resuspended in 1 ml of PBS and incubated in presence of 0.1 mM $FeSO_4$ and 0.2 mM sodium ascorbate for 2 hours at 37°C, while exposed to a 5% CO_2 atmosphere in a cell culture incubator. In order to measure basal levels of lipid peroxides, cell samples were incubated following the same procedure in absence of Fe^{2+}/ascorbate in the incubation medium. The extent of basal and Fe^{2+}/ascorbate-induced lipid peroxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Esterbauer and Cheeseman (14), modified as previously described (15). Basal levels of TBARS were measured fluorimetrically (excitation at 530 nm, emission at 550 nm) while stress-induced levels were measured spectrophotometrically at 532 nm. In both cases freshly diluted malondialdehyde (MDA) bisdimethylacetal was used as standard. TBARS levels were normalized for cell protein determined according to the procedure of Lowry et al. (16) using bovine serum albumin as standard.

Plasmids and cell transfection. The Rous sarcoma virus (RSV) promoter-driven mutant of H-*ras* Leu61, Ser186-encoding plasmid was constructed by insertion of the pZIP H-*ras* (Leu61,Ser186) coding region (1.2 kb BamHI fragment) (17) into the polylinker of pRSV.H20 (18). For transfection experiments K-*ras* transformed E32-4-2 cells were plated in 100 mm dishes at a density of 500,000 cells per dish, in complete medium. Twenty-four hours later the cells were cotransfected by the calcium phosphate technique (19) with 3 μg of pCMV-lacZ and 10 or 30 μg of H-*ras* L61,S186 DNA. Control cells were transfected with 3μg of pCMV-lac Z alone. The amount of DNA in each precipitate was normalized to 33 μg with salmon sperm DNA. After 18 h of treatment with DNA-calcium phosphate

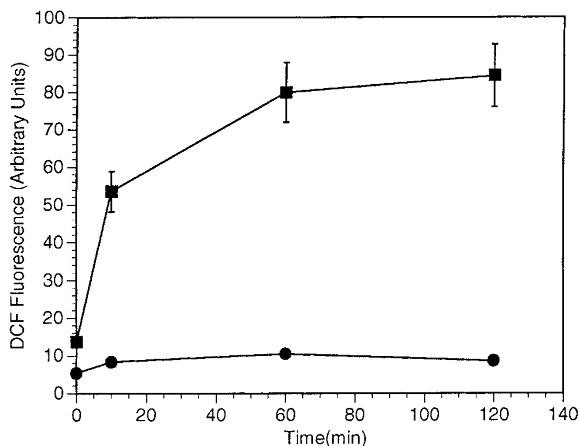

FIG. 1. Time-course of increasing DCF fluorescence induced by Fe^{2+}/ascorbate in NIH 3T3 (■) and K-ras transformed E32-4-2 cells (●). Cells were trypsinized, washed 3 times with PBS, and preincubated for 1 h with DCHF-DA. Then $FeSO_4$ (0.1 mM) and sodium ascorbate (0.2 mM) were added and aliquots of cell samples were removed for DCF fluorescence analysis by FACS at the indicated times, as described under Materials and Methods. Data are means +/− standard error of three separate experiments performed in duplicate.

precipitate, the cells were washed once with PBS and cultured for 48 h in complete medium before harvesting for fluorescent detection of ROS.

Cell viability. Cell viability under oxidative stress conditions, was evaluated by trypan blue exclusion technique.

RESULTS AND DISCUSSION

K-ras transformed NIH 3T3 cells (E32-4-2) show an enhanced resistance to oxidative stress. Susceptibility to oxidative stress in NIH 3T3 cells and their K-*ras* transformed counterpart E32-4-2 cells was tested by measuring the time-course of formation of reactive oxygen species (ROS) in Fe^{2+}/ascorbate stressed cells. On this purpose we used the oxidation sensitive fluorescent probe DCHF-DA; this compound is readily taken up by cells; after uptake intracellular esterases hydrolyze the ester bonds, releasing the deacylated non fluorescent compound 2′,7′dichlorofluorescin (DCFH). This reduced substrate is oxidized by ROS to the fluorescent compound 2′,7′ dichlorofluorescein (DCF) measured by FACS.

To induce oxidative stress, cells were incubated, as described above, with Fe^{2+}/ascorbate (0.1 and 0.2 mM, respectively). The induction of free radicals by $FeSO_4$/ascorbate is due to Fe^{2+} ions, while ascorbate reduces Fe (III) to Fe (II) strongly enhancing the peroxidative potential of ferrum ions (20). The concentrations of Fe^{2+}/ascorbate used were chosen in order to obtain large amounts of ROS (6-fold and 1.6- fold induction after 2 h of incubation, compared to basal levels in NIH 3T3 and E32-4-2 cells, respectively) (fig.1), without significantly impairing cell viability that was higher than 98% in both cell types.

As shown in figure 1, basal levels of ROS, measured as DCF fluorescence after 1 h of preincubation of cells with 10 μM DCHF-DA alone, were 2.5-fold lower in K-*ras* transformed E32-4-2 cells compared to nontransformed NIH 3T3 cells; moreover, stress-induced levels of ROS, measured at the indicated times after Fe^{2+}/ascorbate addition, were 6-10-fold lower in K-*ras* transformed cells. Similar results were obtained when oxidative stress was alternatively induced by H_2O_2 or tert-butylhydroperoxide (data not shown).

Effect of inhibitors of Ras farnesylation on basal and stress-induced levels of ROS in NIH 3T3 and E32-4-2 cells. Preincubation of transformed cells with the inhibitor of HMG-CoA reductase lovastatin (10 μM) reduced the amount of activated $p21^{ras}$ bound to cell membranes

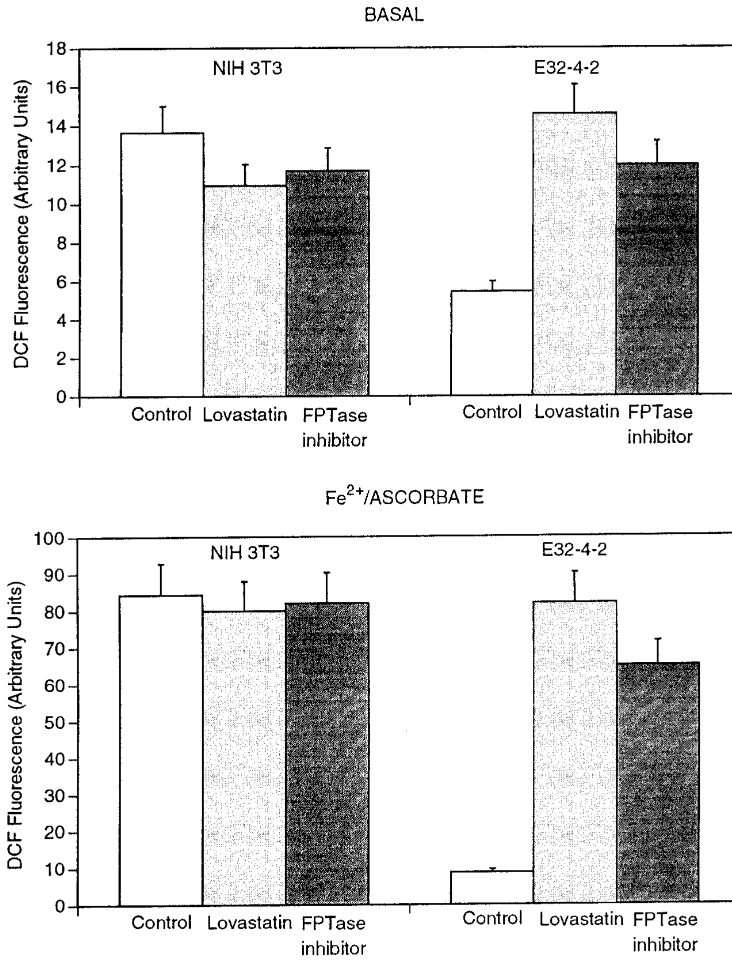

FIG. 2. Effect of inhibitors of Ras processing on basal and Fe^{2+}/ascorbate-induced DCF fluorescence in NIH 3T3 and K-ras transformed E32-4-2 cells. Cells were incubated at 37°C for 24 h with lovastatin (10 μM) or FPTase inhibitor (α-hydroxyfarnesyl) phosphonic acid (100 μM), as indicated in fresh culture medium. Then cells were trypsinized, washed 3 times with PBS, and preincubated for 1 h with 10 mM DCHF-DA at 37°C and kept in DCHF-DA thereafter. Cells were incubated for 2 h at 37°C in the absence (basal levels) and presence of 0.1 mM $FeSO_4$ and 0.2 mM sodium ascorbate prior to DCF fluorescence analysis by FACS, as described under Materials and Methods. Data are means +/− standard error of three separate experiments performed in duplicate.

by 50% (data not shown). Moreover, the selective inhibitor of FPTase (α-hydroxyfarnesyl) phosphonic acid, an analog of the FPTase substrate farnesyl diphosphate, was previously demonstrated to block Ras processing in vivo (21). Both inhibitors of Ras farnesylation led to a complete reversion of the effect of cell transformation either on basal or stress-induced levels of ROS that reached values comparable to that of nontransformed NIH 3T3 cells (fig. 2). Either lovastatin or FPTase inhibitor, (α-hydroxyfarnesyl) phosphonic acid did not significantly affect ROS levels in control NIH 3T3 cells.

Effect of inhibitors of Ras farnesylation on basal and stress-induced levels of lipid peroxides in NIH3T3 and E32-4-2 cells. Since lipid peroxidation is considered as a major mechanism of free radical-induced cell damage, we next examined susceptibility to lipid peroxidation in

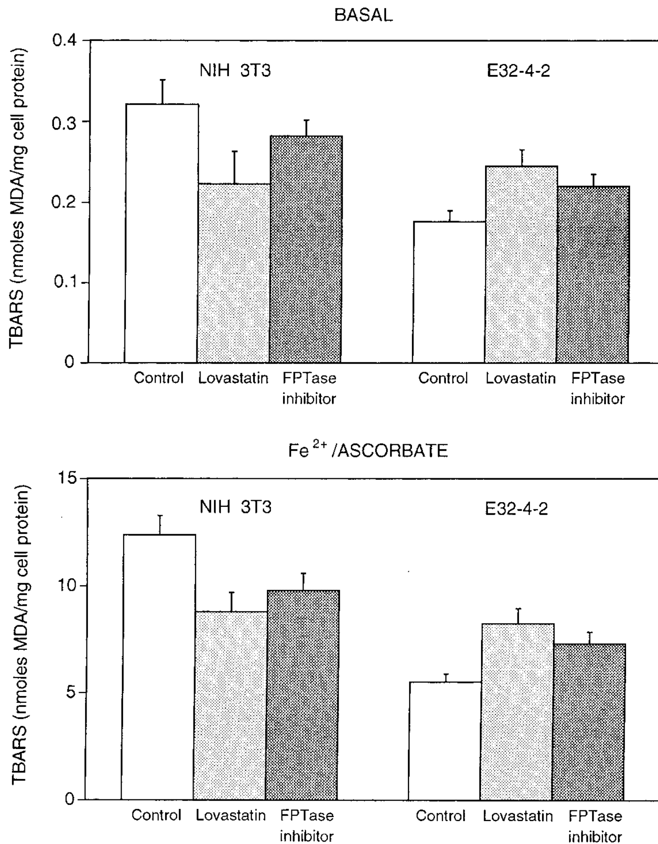

FIG. 3. Effect of inhibition of Ras processing on basal and Fe^{2+}/ascorbate-induced TBARS levels in NIH 3T3 cells and K-ras transformed E32-4-2 cells. Cells were incubated at 37°C for 24 h with lovastatin (10 μM) or FTPase inhibitor (α-hydroxyfarnesyl) phosphonic acid (100 μM), in fresh culture medium as indicated. Then cells were trypsinized, washed 3 times with PBS, and incubated for 2 h at 37°C in the absence and presence of 0.1 mM $FeSO_4$ and 0.2 mM sodium ascorbate in 1 ml PBS. Basal TBARS levels were measured fluorimetrically while Fe^{2+}/ascorbate-induced levels spectrophotometrically, as described under Materials and Methods. Data are means +/− standard error of three separate experiments performed in duplicate.

this model of cell transformation. The K-*ras* transformed E32-4-2 cells show basal and stress-induced TBARS levels significantly lower than those of normal NIH 3T3 cells (fig. 3). Preincubation of E32-4-2 cells with 10μM lovastatin resulted in an increase of basal (40%) and stress-induced (50%) TBARS levels. Analogously (α-hydroxyfarnesyl) phosphonic acid (100μM) treatment of E32-4-2 cells led to an increase of basal (27%) and stress-induced (33%) levels of TBARS.

Transfection of E32-4-2 cells with the transdominant negative mutant of H-ras L61,S186 enhances susceptibility to oxidative stress in these cells. In order to verify the direct involvement of oncogenic *ras* in the enhanced resistance to oxidative stress in K-*ras* transformed E32-4-2 cells compared to their nontransformed counterpart the time-course of ROS formation was measured in E32-4-2 cells in which oncogenic *ras* was inactivated by transfection with the transdominant negative mutant of H-ras L61,S186. This *ras* mutant, which was originally selected by genetic screening of *Saccharomyces Cerevisiae,* has a serine at a position 186 replacing a cysteine that prevents correct attachment of protein to the inner surface of plasma

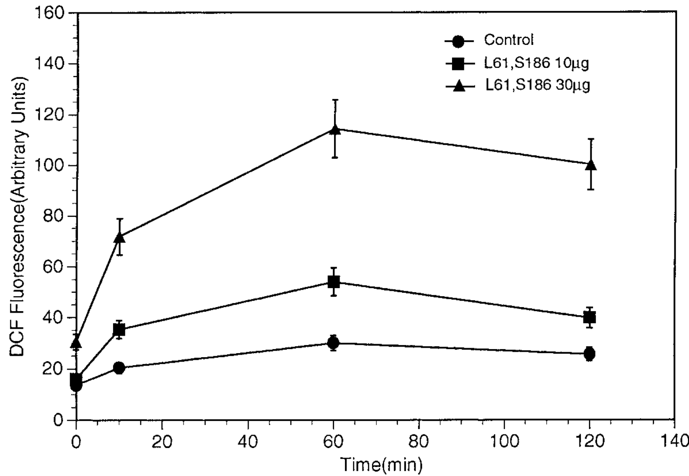

FIG. 4. Time-course of increasing DCF fluorescence induced by Fe^{2+}/ascorbate in E32-4-2 cells transfected with the transdominant negative mutant of H-Ras (L61S186). E32-4-2 cells were cotransfected with 10 or 30 μg of L61,S186 H-Ras and 3 μg of CMV-lacZ as a control plasmid per 100-mm dish, as described under Materials and Methods. Control was transfected with pCMV lacZ alone. For DCF fluorescence measurement, cells were treated as described in the legend to Fig. 1. Values were normalized for β-galactosidase activity. Data are means +/− standard error of three separate experiments performed in triplicate.

membrane and consequently renders the protein nonfunctional. Furthermore the mutant protein has a leucine substituited for glutamine at position 61 that results in a loss of GTPase activity and in 50-fold increase of affinity for GTPase activating protein (22,23). This *ras* mutant has been proposed to inhibit function of $p21^{ras}$ by competition with its cellular effectors (24).

The transfection of K-*ras* transformed E32-4-2 cells with H-*ras* L61,S186, resulted in a dose-dependent increase of ROS levels. In cells treated with 30 μg of H-*ras* L61,S186 DNA, ROS levels reached basal values 2-fold higher and stress-induced values 4-fold higher than those of controls (fig. 4). Similar results were obtained when TBARS levels were measured in E32-4-2 cells transfected with 30 μg of H-ras L61,S186 DNA (data not shown). These data demonstrate that the high resistance to oxidative stress in K-*ras* transformed cells is strictly connected with $p21^{ras}$ activity. Transfection of NIH3T3 cells with H-*ras* L61,S186 failed to affect ROS levels in these cells (data not shown).

It has been previuosly shown a decline in radiation resistance in EJ-*ras* transformed h-osteosarcoma cells after lovastatin treatment (8) and in rat embryo fibroblasts after farnesyl-transferase inhibitor FTI-277 addition (25). At the difference with the previous report our data clearly demonstrate that the inactivation of $p21^{ras}$, induced by these drugs, is responsible for the enhanced stress-induced ROS and lipid peroxide levels in *ras*-transformed cells, thus increasing their radiation response.

In conclusion we suggest that the clinical use of farnesylation inhibitors, because of their ability to enhance susceptibility to oxidative stress, could open new possibilities to improve efficacy of cancer therapy based upon free radical production in *ras*-dependent tumors.

ACKNOWLEDGMENTS

This work was supported in part by the Associazione Italiana Ricerca sul Cancro (A.I.R.C.). We thank M. Berardone for the artwork.

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