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Biochemical Pharmacology 64 (2002) 41–48

Biochemical Pharmacology

Alterations in membrane-bound and cytoplasmic K-ras protein levels in mouse lung induced by treatment with lovastatin, cholestyramine, or niacin: effects are highly mouse strain dependent

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Received 17 September 2001; accepted 3 January 2002

Abstract

Agents that either increase (cholestyramine, CS) or decrease (lovastatin, Lov) *de novo* peripheral cholesterol synthesis may increase (CS) or decrease (Lov) ras protein membrane localization by altering protein prenylation, and potentially have pro- or anti-carcinogenic effects. Male A/J, Swiss, and C57/BL6 mice were treated with 2 or 4% CS, 1% dietary niacin, or 25 mg/kg of Lov three times per week (Lov-3X) or five times per week (Lov-5X). After 3 weeks, serum cholesterol and triglycerides were determined enzymatically. Membrane and cytoplasmic K-ras proteins in lung were determined by immunoprecipitation followed by western blotting with a K-ras specific antibody. Results confirmed the hypothesis only in isolated instances. A/J mice had a significant 30% increase in cytoplasmic K-ras and a 40% decrease in membrane K-ras from Lov treatment, as predicted. C57/BL6 mice had a significant 77% increase in membrane K-ras, as expected from CS feeding. At variance with the hypothesis, Swiss mice had increased levels (3–28%) of membrane K-ras with all treatments (including Lov), and C57/BL6 mice treated with Lov had a 58–78% increase in cytoplasmic K-ras without any reduction in the levels of membrane K-ras. Niacin, predicted to have no effect on ras membrane localization, decreased cytoplasmic K-ras in A/J mice, increased both membrane and cytoplasmic K-ras in Swiss mice, and had no effect in C57/BL6 mice. Results may have differed from those predicted because of strain-dependent differences in response to the cholesterol-lowering agents. A difference in response among the mouse strains suggests that individual genetic differences may alter the effect of hypocholesterolemic agents on K-ras membrane localization, and potentially the risk of ras-dependent cancer. Published by Elsevier Science Inc.

Keywords: Cholesterol; Lovastatin; Cholestyramine; Niacin; Mouse lung; K-ras

1. Introduction

One concern expressed regarding the cholesterol-lowering trials conducted in the 1970s and 1980s was an apparent excess in cancer deaths seen in the intervention group of many of the trials. A published meta-analysis of these trials found a significant ($P < 0.05$) 24% excess of cancer deaths among the treated versus untreated subjects [1]. Lung, colon, and pancreatic cancers were the types reported most frequently in these trials. The K-*ras* proto-oncogene is frequently mutated in mouse and human lung adenocarcinomas. Furthermore, it appears that K-ras protein expression is confined to the alveolar type I and type II cells in lung tissue [2]. Alveolar type II cells are considered to be the progenitor cell for lung adenocarcinoma. Recent results suggest that normal K-ras protein may have a tumor suppressor function in lung, whereas the mutant protein has a positive influence

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Abbreviations: CS, cholestyramine; DMBA, 7,12-dimethylbenz[*a*]anthracene; DTT, DL-dithiothreitol; HMG-CoA reductase, hydroxymethylglutaryl coenzyme A reductase; Lov, lovastatin; Lov-3X, lovastatin (25 mg/kg) three times per week; Lov-5X, lovastatin (25 mg/kg) five times per week; PBS-T, phosphate-buffered saline with 0.1% Tween 20, pH 7.5; PMSF, phenylmethylsulfonyl fluoride.

0006-2952/02/$ – see front matter. Published by Elsevier Science Inc.
PII: S0006-2952(02)01077-8

on tumorigenesis[2] [3,4]. Therefore, study of the effects of lipid-lowering agents on K-ras in lung would be expected to be helpful in trying to explain the increase in cancers of the lung seen in some of the lipid-lowering trials.

We hypothesized that alterations in the membrane-association of the K-ras oncoprotein, induced by the lipid-lowering treatments, may have been responsible for the increased incidence of lung tumors seen in some of the lipid-lowering trials. The supply of farnesyl or geranylgeranyl moieties necessary to anchor the K-ras protein in the cell membrane is regulated largely by the activity of HMG-CoA reductase (hydroxymethylglutaryl-CoA reductase, EC 1.1.1.88). Interventions common in the lipid-lowering trials (e.g. CS resin), which deplete the body of pre-formed cholesterol or its bile acid metabolites, are known to up-regulate HMG-CoA reductase activity in peripheral cells [5,6]. This would increase the supply of farnesyl or geranylgeranyl moieties available for coupling to K-ras protein, and potentially drive more mutant K-ras protein to the cell membrane, where it must be located in order to exercise oncogenic effects [7]. Studies of isolated farnesyltransferase (farnesyl-diphosphate farnesyltransferase, EC 2.5.1.21) *in vitro* have shown that enhanced availability of farnesyl moieties triggers enhanced release of farnesylated protein product, also consistent with our hypothesis [8]. Consistent with this concept, feeding of CS resin has been shown to enhance the yield of chemically induced mammary [9] and colonic [10] tumors in rats. On the other hand, feeding HMG-CoA reductase inhibitors would be expected to reduce prenyl group availability, ras membrane attachment, and mutant ras-related tumorigenesis. Supporting this idea, lovastatin (Lov) or pravastatin were found to have a protective effect against chemically induced lung carcinogenesis in A/J mice [11] and against chemically induced colon carcinogenesis in F344 rats [12]. Both lovastatin and pravastatin are currently in wide clinical use as hypocholesterolemic drugs.

Our experiment was planned to test the hypothesis that hypocholesterolemic agents would have distinct effects on K-ras membrane localization, depending on their effect on *de novo* cholesterol synthesis (CS). CS, an agent that lowers serum cholesterol while enhancing peripheral synthesis of cholesterol in body cells, would be expected to increase the level of farnesyl or geranylgeranyl moieties and to result in a relative increase of membrane-associated K-ras protein. Lov, an inhibitor of HMG-CoA reductase, would be expected to reduce the availability of farnesyl or geranylgeranyl moieties and reduce the level of membrane-associated K-ras protein. Niacin, a hypocholesterolemic agent, which does not affect peripheral tissue cholesterol synthesis [13], would not be expected to affect K-ras membrane localization. Niacin is of interest both as an agent without reported effects on *de novo* cholesterol synthesis, and for its use as a dietary supplement.

This hypothesis was tested in lung tissue of male mice of three different strains, selected based on their differing sensitivity to chemically induced lung tumorigenesis. A/J mice were selected as a sensitive strain, Swiss mice as intermediate, and C57/BL6 mice as a strain relatively resistant to chemically induced lung carcinogenesis.

2. Materials and methods

2.1. Experimental animals and treatments

Male A/J, Swiss, and C57/BL6 mice (6–8 weeks of age) were obtained from the National Cancer Institute at the Frederick Animal Facility breeding colony. After a 1-week adaptation period, they were divided into six treatment groups, with 5 mice of each strain per group. The groups were control, 2% CS (Upshur Smith), 4% CS, 1% niacin (Sigma), lovastatin (Merck) 25 mg/kg, i.p., three times per week (Lov-3X), and lovastatin 25 mg/kg, i.p., five times per week (Lov-5X). The composition of the control diet [14] is shown in Table 1. CS or niacin was substituted for cornstarch in their respective diets. Lov was given by intraperitoneal injection because this route of administration had been shown previously to reduce significantly membrane-associated ras protein in skin tumors of mice [15]. Mice injected with Lov were fed the control diet. Animals had free access to diets and to acidified tap water throughout the experiment, and were treated for 3 weeks. Body weights and food intakes were recorded weekly. Animal care was provided in accordance with the procedures in the NIH Guide for the Care and Use of Laboratory Animals.

Mice were anesthetized by light carbon dioxide inhalation. Blood was obtained by cardiac puncture, and the serum was separated and frozen. Lungs were removed and frozen in liquid nitrogen and stored at −80° until analysis.

2.2. Serum cholesterol and triglyceride analysis

Sera were analyzed for total cholesterol and triglyceride content by commercial enzyme kits.

Table 1
Composition of the control diet

Ingredient	g/kg
Casein	140
Cornstarch	465.692
Dextrose	155
Sucrose	100
Cellulose	50
Soybean oil	40
t-Butylhydroquinone	0.008
Salt mix [13]	35
Vitamin mix [13]	10
L-Cystine	1.8
Choline bitartrate	2.5

[2] Ramakrishna G, Sithanandam S, Smith GT, Fornwald LW, Masuda A, Takahashi T, Anderson LM. Manuscript submitted for publication.

2.3. Lung tissue preparation

Whole lungs were homogenized in hypotonic buffer [10 mM Tris, 5 mM EDTA, 150 mM NaCl, 10% sucrose with 30 μg/mL of aprotinin (Sigma), and 1 μmol/mL of PMSF (Sigma)] in a glass vessel immersed in ice with a motor-driven teflon pestle. One portion of this homogenate was set aside for ultracentrifugation, while the remainder was prepared as a whole cell lysate. To prepare the whole cell lysate, a 1/10th volume of a 10× buffer containing Triton X-100 (Sigma), SDS (BioRad), and sodium deoxycholate (Sigma) was added, resulting in final concentrations of 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. After a 2-hr period on ice, this whole cell lysate was centrifuged at 14,000 rpm in a microfuge (Eppendorf) for 10 min at 4°. The supernatant was removed, frozen in liquid nitrogen, and stored at −70° until analysis.

The other aliquot was centrifuged in an XL-90 ultracentrifuge (Beckman) at 101,162 *g* using a 50 Ti rotor at 4° for 1 hr. The supernatant, representing the cytoplasmic fraction, was removed and frozen in liquid nitrogen, and stored at −70° until analysis. The pellet was washed, then resuspended in isotonic buffer (10 mM Tris–HCl, 250 mM sucrose, 50 mM NaCl, 30 μg/mL of aprotinin, 1 μmol/mL of PMSF), and the suspension was centrifuged again as above.

The resulting pellet was washed twice in isotonic buffer, then homogenized in a glass vessel immersed in ice with a motor-driven teflon pestle. Membrane lysis buffer (50 mM Tris, 0.1% SDS, 150 mM NaCl, 1% Triton X-100 with 60 μg/mL of aprotinin and 2 μmol/mL of PMSF) was used in this homogenization. The membrane fraction was allowed to sit on ice for 2 hr, and then was centrifuged for 10 min at 14,000 rpm in a microfuge (Eppendorf) at 4°. The supernatant, representing the membrane fraction, was frozen and stored as described above until analysis.

2.4. Ras immunoprecipitation

Total protein in each fraction was determined using the BCA kit (Pierce). Total (H-, N-, and K-) ras protein was immunoprecipitated with 30 μL of agarose beads coupled to a v-H-ras antibody (Calbiochem) in an overnight incubation at 4°. Experiments conducted on each batch of the antibody-coupled beads showed equal recovery of ras protein with 20, 30, or 40 μL of beads. Optimal protein amounts to use in immunoprecipitation for each subcellular fraction were determined by standard curve experiments. These optimal amounts were 200 μg for whole cell lysate, 500 μg for the cytoplasmic fraction, and 100 μg for the membrane fraction. After overnight immunoprecipitation, the beads were isolated by centrifugation for 20 sec in a microcentrifuge (Eppendorf) at 14,000 rpm at room temperature, and washed three times. After the removal of the final wash solution, 30 μL of loading buffer [100 mM Tris–HCl, 400 mM DTT (Promega), 20% (v/v) glycerol (Life Technologies), 4% SDS, 0.002% (w/v) bromphenol blue (Sigma)] was added.

2.5. Gel electrophoresis and western blotting

Tubes were boiled for 13 min to denature proteins and centrifuged (in an Eppendorf microcentrifuge for 20 sec at full speed) to precipitate the beads; then 15 μL of the supernatant was loaded onto a 12% polyacrylamide Tris-glycine 8 × 10 cm, 1-mm thick commercial pre-cast gel (Invitrogen). The gel was subjected to electrophoresis at 120 V for 2 hr in 25 mM Tris-base, 192 mM glycine, 0.1% SDS running buffer. The separated protein bands were transferred to an Amersham Hybond ECL membrane (Amersham Pharmacia Biotech) with a Novex XCell II blot module electroblotter (Invitrogen) at 22 V for 1 hr. The transfer buffer contained 12 mM Tris base, 96 mM glycine, and 20% (v/v) methanol. The membrane was washed with PBS-T, and then blocked overnight with 3% (w/v) BSA (Sigma) in PBS-T. Following three 10-min washes in PBS-T, the membrane was incubated at room temperature for 2 hr with the primary antibody, a mouse anti-c-K-ras monoclonal antibody (Calbiochem) diluted 1:10,000 in 3% (w/v) powdered skim milk in PBS-T. After three additional 10-min washes in PBS-T, the membrane was incubated for 1 hr at room temperature with the secondary antibody, an anti-mouse IgG linked to horseradish peroxidase (Amersham Pharmacia Biotech). The secondary antibody was diluted 1:2000 in 3% (w/v) dry skim milk in PBS-T. Preliminary studies found the K-ras antibody to also detect H-ras, but at twenty times less affinity compared with K-ras. The antibody did not appear to react at all with N-ras. Further details of preliminary studies to establish optimal conditions for immunoprecipitation and antibody specificity have been published previously [16].

2.6. Band visualization by chemiluminescence

K-ras protein bands were visualized by incubation of the membrane (after washing three times as above) with freshly mixed ECL reagent (Amersham Pharmacia Biotech) according to the instructions provided with the ECL kit. Following a pre-flash according to the protocol of the manufacturer with a Sensitize preflash unit (Amersham Pharmacia Biotech), membranes were allowed to expose ECL Hyperfilm (Amersham Pharmacia Biotech) in an autoradiography cassette for 15 sec to 3 min. Exposure time was adjusted to maintain samples within the linear response range as established in earlier standard curve experiments. Films were processed in a Kodak M35A X-OMAT processor (Eastman Kodak).

2.7. Densitometric scanning

Bands of interest on the developed films were scanned with a Molecular Dynamics Personal Densitometer

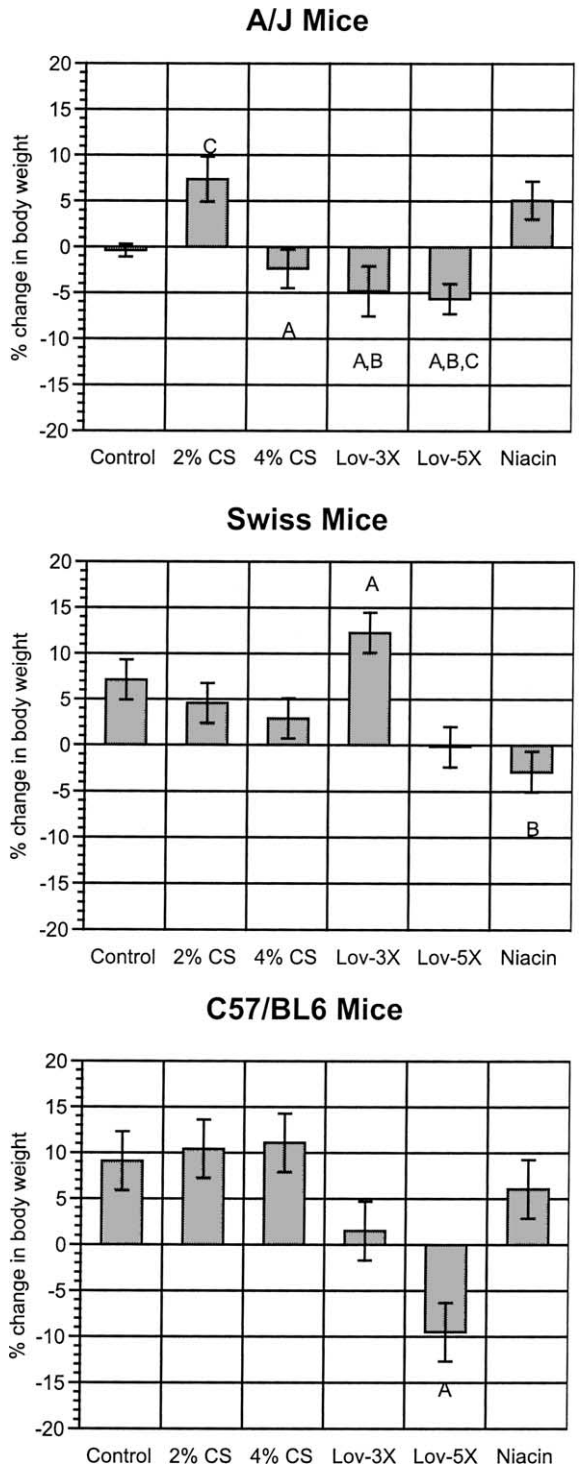

Fig. 1. Percent changes in body weight during the 3-week experiment for male A/J, Swiss, and C57/BL6 mice. Treatments include control, 2 or 4% cholestyramine (CS), lovastatin 25 mg/kg by intraperitoneal injection three (Lov-3X) or five (Lov-5X) times per week, or 1% niacin. Values are means ± SEM. Each bar represents the mean of values from 5 animals, except for the Lov-3X group of Swiss mice, where N = 4 due to an unavailable initial weight for one mouse. Unless otherwise noted, statistically significant differences were detected by ANOVA (for A/J and Swiss mice) or the Kruskal–Wallis test (C57/BL6 mice) followed by pairwise Tukey or Dunn testing. Mean initial weights of the various treatment and control groups of mice ranged from 24.8 to 27.2 g (A/J), from 26.0 to 28.4 g (Swiss), or from 24.3 to 25.0 g (C57/BL6). None of the differences in mean initial body weights were significantly different among treatment groups within a mouse strain. Explanation of symbols: upper panel (A/J mice): (A) differs significantly from the 2% CS group ($P < 0.05$). (B) Differs significantly from the niacin group ($P < 0.05$). (C) Differs significantly from the control group ($P < 0.05$, by *t*-test only). Middle panel (Swiss mice): (A) differs significantly from the niacin group ($P < 0.05$). (B) Differs significantly from the control group ($P < 0.01$, by *t*-test only). Lower panel (C57/BL6 mice): (A) differs significantly from the 2 or 4% CS groups ($P < 0.01$, Kruskal–Wallis test) or from the control ($P < 0.01$, by *t*-test only).

equipped with Image Quant v. 3.3 software (Molecular Dynamics).

2.8. Comparisons between groups

The amounts of total, membrane, and cytoplasmic K-ras protein from treated samples were compared with control samples run on the same blot. Results from each treatment within a given mouse strain were compared to the same five control samples that were run on each blot. Thus, the controls served as a standard of comparison for each treatment.

For K-ras membrane to cytoplasm ratios, the densitometric values of one animal's membrane and cytoplasmic K-ras protein, run side by side on the same blot, were expressed as a ratio, correcting for protein concentration. Thus, each ratio was dependent upon two densitometric values from the same blot. For example, differences in exposure or completeness of transfer between blots would be eliminated since these factors would affect both the numerator and denominator values of the resulting ratio to the same extent. Therefore, these ratios could be compared between blots.

2.9. Statistical analysis

Statistical analysis of the densitometric values was performed using GraphPad InStat v. 3.01 (GraphPad Software). Comparisons were made by ANOVA for multiple groups (for normally distributed data) or by the Kruskal–Wallis test (for data not normally distributed), followed by the Tukey or Dunn pairwise tests, respectively. A two-tailed *t*-test was used to compare two groups that had comparison samples run on the same gel. Differences were considered statistically significant when P values were less than 0.05.

3. Results

3.1. Body weights

To investigate the possibility of indirect toxic effects of the treatments, body weight changes over the 3-week study [expressed as weight gained (or lost) as a percentage of initial weight] were calculated (Fig. 1). These data were analyzed for statistical significance by ANOVA (or non-

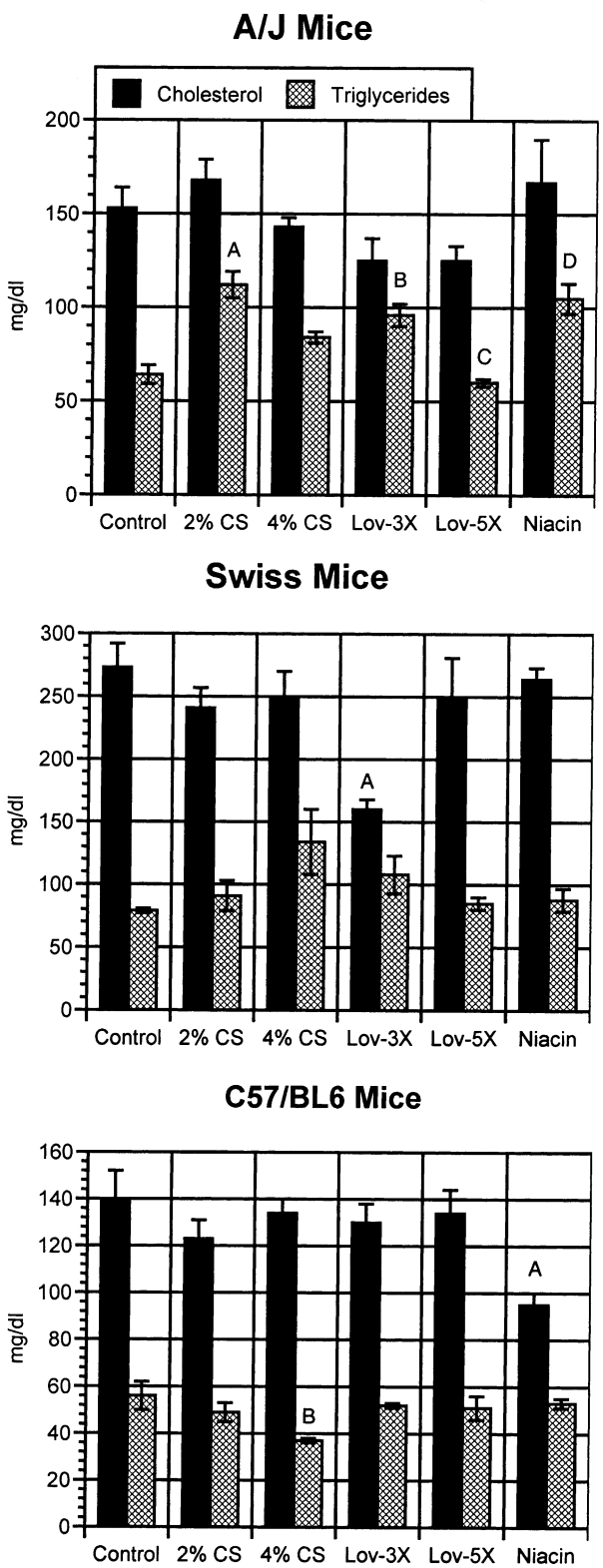

Fig. 2. Serum cholesterol and triglycerides in male A/J, Swiss, and C57/BL6 mice. Group identifications are the same as in Fig. 1. Values are means ± SEM. Each bar represents the mean of values from 5 animals, except for the Lov-5X group of C57/BL6 mice, where cholesterol and triglycerides were determined in only 4 mice. Explanation of symbols: upper panel (A/J mice): (A) differs significantly from the control ($P < 0.001$), 4% CS ($P < 0.05$), and Lov-5X ($P < 0.001$) groups. (B) Differs significantly from the control ($P < 0.01$) and Lov-5X ($P < 0.01$) groups. (C) Differs significantly from the Lov-3X ($P < 0.01$), niacin ($P < 0.001$), and 2% CS ($P < 0.001$) groups. (D) Differs significantly from the control ($P < 0.001$) and Lov-5X ($P < 0.001$) groups. Middle panel (Swiss mice): (A) differs significantly from the control ($P < 0.01$), 4% CS ($P < 0.05$), Lov-5X ($P < 0.05$), and niacin ($P < 0.01$) groups. Lower panel (C57/BL6 mice): (A) differs significantly from the control ($P < 0.01$), 4% CS ($P < 0.05$), and Lov-5X ($P < 0.05$) groups. (B) Differs significantly from the control ($P < 0.05$) and niacin ($P < 0.05$) groups.

parametric Kruskal–Wallis testing) followed by pairwise comparisons with the Tukey test (or nonparametric Dunn test) when the overall ANOVA value was statistically significant. In a few instances, visual inspection of the means and standard errors suggested that other meaningful differences might exist (i.e. means ± SEMs of two groups did not overlap). In these isolated cases, *t*-tests were done to compare these two groups directly. These *t*-test comparisons are identified explicitly in the figure legends. Changes in body weight varied considerably based on both mouse strain and treatment. Control A/J mice did not gain weight over the course of the study, whereas 2% CS and niacin caused weight gain on the average, and 4% CS and both Lov doses were associated with an average decrease in body weight, with some differences of statistical significance among these groups (see legend to Fig. 1). In the Swiss mice, Lov-3X enhanced weight gain and niacin caused weight loss. Lov-5X caused significant weight loss in the C57/BL6 mice, with a suggestion of suppressed weight gain by Lov-3X. Thus, Lov appeared to cause weight gain suppression or weight loss in four of five groups, with weight gain in Swiss mice given Lov-3X as an exception. The effects of 4% CS and niacin were strain-specific.

3.2. Serum cholesterol and triglycerides

The treatments had a significant hypocholesteromic effect in two instances (Fig. 2). An approximate 40% average decrease in serum cholesterol occurred in Swiss mice given Lov-3X, and niacin caused a decrease of about 33% on the average in C57/BL6 mice. Swiss mice had serum cholesterol values considerably higher than in the other two strains (control mean of 273 mg/dL vs 153 or 139 mg/dL for A/J and C57/BL6 mice, respectively).

Serum triglycerides were reduced significantly from control values in only one case, in C57/BL6 mice where 4% CS treatment resulted in a 33% reduction compared with control. Statistically significant differences between groups were also found among the A/J mice (Fig. 2), but none had a serum triglyceride level significantly lower than the control value.

3.3. Total, membrane, and cytosolic K-ras protein

Representative western blots of total, membrane, and cytosolic K-ras protein are shown in Fig. 3. Quantitative results are presented in Fig. 4, as a percent of the control mean for samples run on the same blot. Membrane to

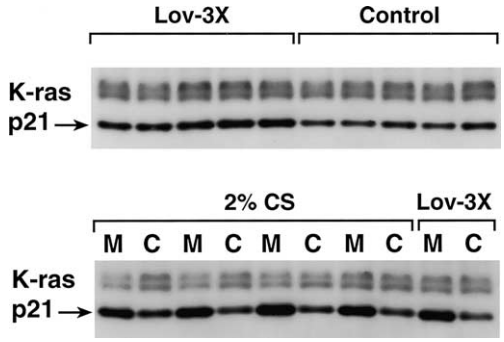

Fig. 3. Upper panel: representative data of a western blot of immunoprecipitated K-ras protein in a total lung tissue lysate. Lov-3X = treated by lovastatin (25 mg/kg) 3 times per week. The upper band is the mouse immunoglobulin light chain. Lower panel: representative western blot of immunoprecipitated K-ras protein from membrane (M) and cytoplasmic (C) fractions of lung tissue. Membrane and cytoplasmic samples from each animal were run side by side on the same gel. 2% CS = 2% cholestyramine diet; Lov-3X = lovastatin (25 mg/kg) 3 times per week treatment. The upper band is the mouse immunoglobulin light chain. Blots comparing total, membrane, or cytosolic K-ras protein with control samples (as in the upper panel) were done for each treatment and mouse strain, amounting to a total of 45 blots. Some blots were repeated a second time to confirm results or if the first blot was technically questionable (e.g. possible poor transfer). Membrane to cytoplasm ratio blots, as in the lower panel, were done for each mouse strain and treatment, for a total of 15 blots.

cytoplasm ratios are also shown as a percent of the control membrane to cytoplasm ratio for each mouse strain. A variety of apparent strain-specific effects was observed. In A/J mice, CS treatment was associated with a minimal increase in total K-ras protein; the 30% increase (4% CS only) in cytosolic protein was significant. There were significant increases in total K-ras protein after both doses of Lov, due entirely to increases in cytosolic protein; both membrane levels and the membrane/cytosol ratio of K-ras protein were reduced. Niacin, on the other hand, had no effect on total K-ras, but caused a significant reduction in the cytosolic protein, with non-significant increases in membrane protein and the membrane/cytosolic protein ratio.

In the Swiss mice, CS treatment was again associated with small increases in total K-ras protein; the increase in membrane K-ras protein was significant for 4% CS. Lov-3X led to a significant, 50% increase in total K-ras protein, due entirely to an increase in the membrane. An increase after niacin feeding was apparent in both membrane and cytosolic protein.

The most marked effects were seen in the C57/BL6 mice. There were increases in total, membrane, and cytosolic K-ras protein and in the membrane/cytosolic ratio after both doses of CS. Most striking was the 60–120% increase in total and cytosolic K-ras protein observed after both Lov doses, with corresponding significant decreases in the membrane/cytosolic ratio.

We considered whether any of the observed effects might be an indirect result of toxicity of the compounds. Inspection of these body weight data revealed that groups with the largest amount of weight loss (the Lov-treated mice) generally had higher total ras levels. Could some of the effects seen with Lov treatment or other treatments be due to general toxicity of the compounds as indicated by weight loss in the animals? If this were the case, in treatment groups with a mean weight loss, it would be expected that levels of total, membrane, and cytosolic K-ras protein, as well as the membrane to cytoplasm ratios in individual mice should correlate with the degree of weight loss in those animals. To attempt to address this in a quantitative manner, K-ras protein values and membrane to cytoplasm ratios for individual animals in groups having mean weight loss were correlated with their individual percent change in body weight by linear regression. In A/J and Swiss mice, treatments producing weight loss (e.g. 4% CS, Lov-3X, Lov-5X in A/J mice) were considered both combined together and separately by treatment. To facilitate comparison of results of different blots in the same regression, the densitometric value for each animal was expressed as a percent of the mean of the control group values for that blot. With one exception, the results of these linear regressions showed no relationships of statistical significance between total, membrane, or cytoplasmic K-ras protein quantity (or K-ras membrane to cytoplasm ratios) and percent change in body weight in any of the mouse strains for mice in treatment groups having a mean weight loss (result not shown). The exception was the niacin group in the Swiss mice, where membrane K-ras was significantly negatively correlated with changes in body weight ($r = -0.9640$, $P = 0.0081$). The increase in membrane K-ras seen in the Swiss mice fed niacin was of a modest magnitude (about 25%) and balanced by an increase in cytoplasmic ras of similar magnitude. Therefore, the biological significance of the correlation between membrane K-ras and body weight in these mice is uncertain.

4. Discussion

The results of this study confirm that drugs utilized to lower serum cholesterol in people can alter the total amount and membrane localization of K-ras protein in the lungs of mice. However, the results were much more complex than predicted, and in general did not correlate with changes in serum cholesterol. The only strong effect on serum cholesterol was a 40% decrease in Lov-3X-treated Swiss mice. This was associated with an increase in total and membrane K-ras protein, rather than the expected decrease. Larger increases in total K-ras after Lov treatment were seen in C57/BL6 mice, where no change in serum cholesterol was noted. A/J mice also experienced an increase in K-ras protein in lung after Lov treatment, but in this strain the amount in the membrane decreased, as was predicted by the hypothesis.

The decrease in membrane-associated K-ras protein found in A/J mice in our study is of particular interest

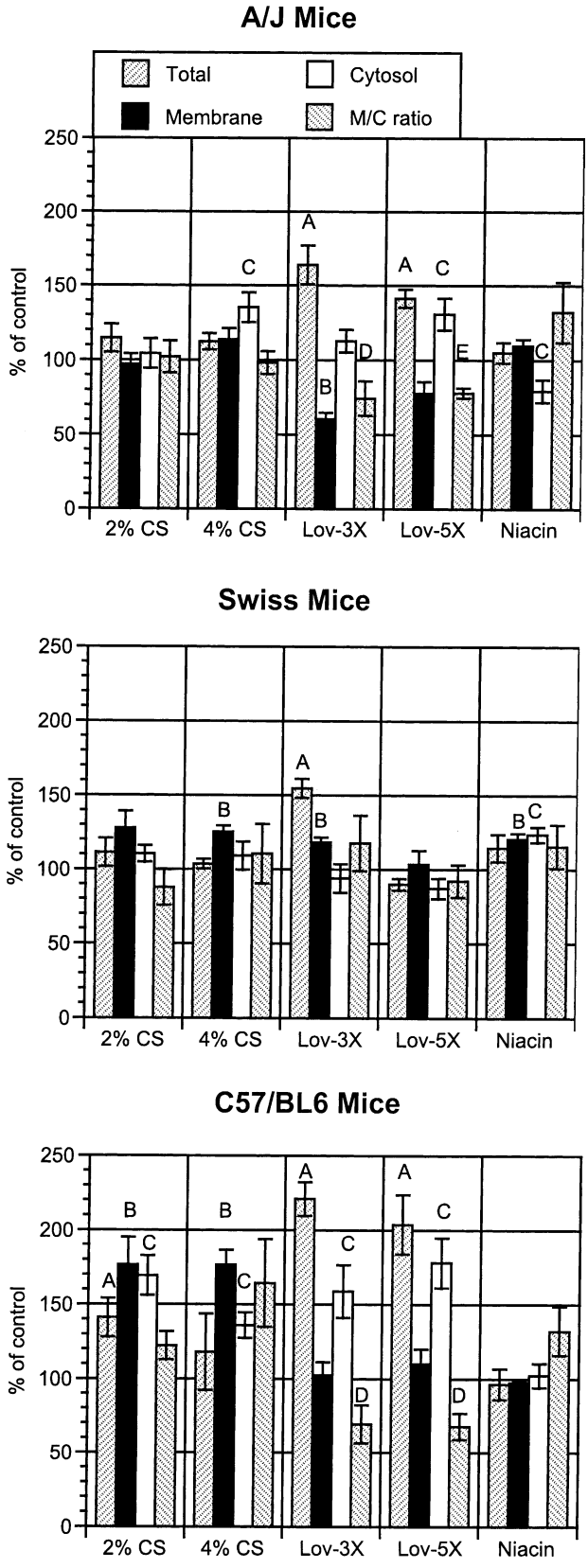

Fig. 4. Graphical representation of total, membrane, and cytoplasmic K-ras protein and K-ras protein membrane to cytoplasm ratios, expressed as a percent of control. Values are means ± SEM. Group identifications are the same as in Fig. 1. Each bar represents the mean of values from 5 animals, except for all values for the A/J mice in the 2% CS group and the C57/BL6 mice in the Lov-5X group, where each bar represents the mean of values from 4 animals. Control group membrane to cytoplasm ratios (mean ± SEM) were 9.97 ± 0.75 (A/J mice), 14.47 ± 1.03 (Swiss mice), and 10.96 ± 1.71 (C57/BL6 mice). Explanation of symbols above bars: top panel (A/J mice): (A) total K-ras protein differs significantly from control, $P < 0.01$ (for Lov-3X) or $P < 0.001$ (for Lov-5X). (B) Membrane K-ras protein differs significantly from control, $P < 0.05$. (C) Cytoplasmic K-ras protein differs significantly from control, $P < 0.05$. For A/J mice the overall ANOVA for the membrane to cytoplasm ratios was statistically significant, but the individual pairwise comparisons were not. Therefore, individual *t*-tests were done to compare groups that by inspection appeared to differ significantly (error bars did not overlap control). These comparisons showed: (D) membrane to cytoplasm ratio differs significantly from the niacin group ($P < 0.05$, *t*-test). (E) Membrane to cytoplasm ratio differs significantly from the control group ($P < 0.05$, *t*-test with Welch correction). Middle panel (Swiss mice): (A) total K-ras differs significantly from the control group ($P < 0.0005$). (B) Membrane K-ras differs significantly from the control group, $P < 0.001$ (for 4% CS), $P < 0.005$ (for Lov-3X), and $P < 0.05$ (for niacin). (C) Cytoplasmic K-ras differs significantly from the control group, $P < 0.01$. Bottom panel (C57/BL6 mice): (A) total K-ras protein differs significantly from the control, $P < 0.05$ (for 2% CS), $P < 0.0005$ (for Lov-3X), and $P < 0.005$ (for Lov-5X). (B) Membrane K-ras protein differs significantly from the control, $P < 0.01$ (for 2% CS) and $P < 0.005$ (for 4% CS). (C) Cytoplasmic K-ras protein differs significantly from the control, $P < 0.05$ (for 2% CS, 4% CS, and Lov-3X) or $P < 0.01$ (for Lov-5X). (D) Membrane to cytoplasm ratio differs significantly from that of the 4% CS group, $P < 0.01$ (for Lov-3X) or $P < 0.05$ (for Lov-5X).

in view of the reported protective effect of Lov fed at 40, 160, or 400 ppm in the diet on chemical carcinogenesis in A/J lungs [11]. Because of differences in the route of administration, it is not possible to precisely compare the doses in that published study with those of our study. For example, the intraperitoneal administration of Lov in our study would be expected to produce higher peak blood levels than comparable doses given orally. A rough comparison may be made, however, if food consumption and body weight data for the A/J mice in our study (these data are not given in Ref. [11]) are used to convert dietary doses of Lov (in ppm) to mg/kg/day. Using an average daily food consumption of 2.7 g/day, and an average body weight of 26.4 g, the 40, 160, and 400 ppm Lov diets would provide 4.1, 16.4, and 40.9 mg/kg/day of Lov, respectively. This compares to daily doses in our study of 10.7 mg/kg/day (Lov-3X) or 17.9 mg/kg/day (Lov-5X). Regardless of the exact quantitative validity of the comparison of the doses of Lov in our study versus the published chemoprotection study [11], a protective effect of Lov is suggested in both studies. The large majority of chemically induced lung tumors in A/J mice contain a mutated K-*ras* gene [17,18], and our data suggest that reduction of the level of mutant K-ras protein in the membrane may be one mechanism by which Lov may reduce lung tumorigenesis.

Serum cholesterol levels are determined largely by liver synthesis. Effects of Lov on HMG-CoA reductase in lung have not been reported but could be dissimilar from those in liver, and could differ among strains. A comparative study of cholesterol metabolism in 14 different inbred mouse strains documented a 24-fold variation in basal levels of hepatic HMG-CoA reductase among strains

[19], and we observed much higher levels of serum cholesterol in control Swiss mice compared with A/J and C57/BL6 mice. In addition to the direct down-regulatory effect of Lov, compensatory increases in HMG-CoA reductase enzyme synthesis and activity have been described for liver [20] and skin [21] in mice. It is clear that a detailed study of the biochemical effects of Lov and other agents targeting protein prenylation should be carried out in the lungs of mice of various strains and in other species.

CS was predicted to increase membrane K-ras protein due to greater availability of prenyl groups in mouse lung by enhanced *de novo* cholesterol synthesis, and this was borne out in the Swiss mice, where a modest increase occurred, not associated with an increase in total K-ras. In C57/BL6 mice, CS led to an increase in total K-ras protein, with slightly more of this increase being present in the membrane fraction than in the cytosol, although both were increased. CS feeding has been found to increase sterol synthesis in the lungs of both rats [5] and guinea pigs [6], suggesting that a larger pool of prenyl moieties would be available.

Although some of the treatments had apparent toxicity, as manifested by weight loss, weight loss effects were not predictive of and did not correlate with the observed differences in K-ras protein quantity and intracellular distribution. Rather, the observed alterations in K-ras appear to be specific pharmacological effects of the treatments. In many cases, however, these effects did not correlate with the original hypotheses and were not associated with changes in serum cholesterol. The results suggest that the underlying phenomena are more complex than we had originally envisioned.

In summary, the results of our study, while limited by lack of information on the effects of the agents on lung biochemistry, show clearly that drugs given to people for the purpose of altering serum cholesterol can have a pronounced effect on the total amount and intracellular localization of an important cancer gene product in lungs of mice. The marked strain differences suggest a major genetic component that could also pertain in humans. The mechanisms, genetics, and cancer-risk implications of these effects need further study, not only with regard to cholesterol-altering agents but also in the context of farnesyltransferase inhibitors being employed in cancer therapy trials [22]. Our data establish a model system for further pursuit of these questions.

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