Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 393–403
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Treatment with lovastatin, cholestyramine or niacin alters K-ras membrane association in mouse lung in a strain-dependent manner: results in females

Richard J. Calvert[a,b,*], Shirley Tepper[c], Bhalchandra A. Diwan[d], Lucy M. Anderson[b], David Kritchevsky[c]

[a]*Division of Research and Applied Technology, Office of Nutritional Products, Labeling and Dietary Supplements, Center for Food Safety and Applied Nutrition, US Food and Drug Administration, College Park, MD 20740, USA*
[b]*Laboratory of Comparative Carcinogenesis, National Cancer Institute at Frederick, Building 538, Rm. 227, Frederick, MD 21702, USA*
[c]*The Wistar Institute, Philadelphia, PA 19104, USA*
[d]*Basic Research Program, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD 21702, USA*

Received 3 September 2002; accepted 28 March 2003

Abstract

Hypocholesterolemic drugs may themselves increase (cholestyramine, CS) or decrease (lovastatin, Lov) peripheral tissue *de novo* cholesterol biosynthesis. This will alter the abundance of prenyl groups and potentially increase (CS) or decrease (Lov) K-ras membrane localization, with possible pro- or anti-carcinogenic effects (K-*ras* is a proto-oncogene frequently mutated in lung cancer). Female A/J, Swiss, and C57BL/6 mice were fed 2 or 4% CS, 1% niacin, or injected with Lov three (Lov-3×) or five (Lov-5×) times per week. After three weeks, serum cholesterol and triglycerides were determined enzymatically. Total, membrane, and cytoplasmic K-ras proteins were determined in lung homogenates by immunoprecipitation followed by Western blotting with a K-ras specific antibody. CS feeding increased membrane K-ras as hypothesized in A/J and C57BL/6 mice, but had no effect in Swiss mice. Lov failed in all three strains to reduce membrane K-ras, and resulted in an increase in total K-ras in A/J and C57BL/6 mice, while again lacking effect in Swiss mice. Niacin had no effect on K-ras protein in any mouse strain. These results differ from our published results for male mice of the same strains, particularly for A/J mice. Increased amounts of K-ras protein in the membrane fraction of A/J females (but not males) treated with either Lov or CS imply that if K-ras were to become mutated, CS could result in increased lung tumorigenesis and Lov would be less likely to be protective in females. In the light of these data, both sexes should be included in future animal and human chemoprevention trials.

Keywords: Cholesterol; Cholestyramine; K-ras; Lovastatin; Lung; Mouse; Niacin; Sex difference

1. Introduction

Lung cancer is the leading cause of cancer death for both men and women in the United States [1]. While tobacco use accounts for the vast majority of lung cancer cases, concern has also been expressed regarding possible cancer-enhancing effects of some of the lipid-lowering drugs used in the cholesterol-reduction trials conducted in the 1970s and early 1980s. Indeed, a published meta-analysis of these trials found a significant ($P < 0.05$) 24% excess of cancer deaths in the treated vs. untreated subjects [2]. Lung, colonic, and pancreatic cancers were most commonly reported in those trials which reported cancer type.

The K-*ras* gene is frequently mutated in both human and mouse lung adenocarcinoma. In lung, its expression is confined to types I and II alveolar cells [3]. Type II cells are considered to be a cell of origin for lung adenocarcinomas. Wild-type K-ras may function as a tumor suppressor [4–7], while a mutated K-ras enhances tumorigenesis. Because the K-ras protein requires post-translational attachment of a prenyl group to be anchored in its active position in the cell membrane [8], we decided to examine

* Corresponding author. Tel.: +1-301-846-1246; fax: +1-301-846-5946.
E-mail address: calvert@mail.ncifcrf.gov (R.J. Calvert).

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

0006-2952/$ – see front matter
doi:10.1016/S0006-2952(03)00211-9

whether various cholesterol-lowering drugs, which might affect prenyl group availability, would alter K-ras protein membrane attachment in the lungs of female mice. Previous work had suggested that such alterations occurred in a strain-specific manner in male mice [9], and we wished to extend these observations to females. This comparison is of interest as it has been suggested that women may be more susceptible to lung cancer than men [5,10,11], and particularly that they may differ from men in their response to dietary interventions intended to reduce lung cancer risk [11,12].

We examined whether the membrane localization of the K-ras protein would be altered by different lipid-lowering drugs. 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 hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase, E.C. #1.1.1.88). Interventions common in the lipid-lowering trials [e.g. cholestyramine (CS) resin] which deplete the body of preformed cholesterol or its bile acid metabolites are known to upregulate HMG-CoA reductase activity in peripheral cells [13,14]. This would increase the supply of farnesyl or geranylgeranyl moieties available for coupling to K-ras protein, and potentially drive more K-ras protein to the cell membrane, where it must be located in order to exercise signaling and oncogenic effects [8]. If the K-*ras* gene were to become mutated, an increase in active, membrane-associated K-ras protein would be expected to promote lung cancer development. Consistent with this concept, feeding of CS resin has been shown to enhance the yield of chemically-induced mammary [15] and colonic [16] tumors in rats. In contrast, reducing cholesterol by treatment with 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/JNCr (abbreviated hereafter A/J) mice [17] and against chemically-induced colon carcinogenesis in F344 rats [18]. Both Lov 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, a drug which 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 result in a relative increase of membrane-associated K-ras protein. Lov, an inhibitor of HMG-CoA reductase, would be expected to decrease the concentration 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 [19], 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 female 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 C57BL/6NCr (C57BL/6) mice as a strain relatively resistant to chemically-induced lung carcinogenesis. Results of this experiment are also contrasted with those of our earlier study in male mice of the same three strains [9].

2. Materials and methods

2.1. Experimental animals and treatments

Female A/J, Swiss/NCr (Swiss), and C57BL/6 mice were obtained at six to eight weeks of age from the National Cancer Institute at Frederick Animal Production Area breeding colony. After a one-week adaptation period, they were divided into six treatment groups, with five mice of each strain per group. The groups were control, 2% CS (Upshur Smith), 4% CS, 1% niacin (Sigma), Lov (Merck) 25 mg/kg intraperitoneally (i.p.) three times per week (Lov-3×), and Lov 25 mg/kg i.p. five times per week (Lov-5×). The composition of the control diet [20] is shown in Table 1. CS or niacin was substituted for corn starch in their respective diets. Lov was given by i.p. injection because this route of administration had been shown previously to reduce significantly the membrane-associated ras protein in skin tumors of mice [21]. Although Lov is given orally in humans, we wished to use a dosage and route of administration known to affect ras membrane localization in mice. Mice injected with Lov were fed the control diet. Animals had free access to diets and to acidified tap water throughout the experiment. Animals were treated for three 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. The NCI-Frederick animal facility and its animal program are accredited by the American Association for Accreditation of Laboratory Animal Care.

Table 1
Composition of the control diet

Ingredient	Composition (g/kg)
Casein	140
Cornstarch	465.692
Dextrose	155
Sucrose	100
Cellulose	50
Soybean oil	40
t-Butylhydroquinone	0.008
Salt mix [20]	35
Vitamin mix [20]	10
L-Cystine	1.8
Choline bitartrate	2.5

Mice were anesthetized by light carbon dioxide inhalation. Blood was obtained by cardiac puncture, serum 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.

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 aprotinin (Sigma) and 1 μmol/mL 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 one-tenth volume of a 10X buffer containing Triton X100 (Sigma), SDS (BioRad), and sodium deoxycholate (Sigma) was added, resulting in final concentrations of 1% Triton X100, 0.1% SDS and 0.5% sodium deoxycholate. After a 2-hr period on ice, this whole cell lysate was centrifuged at 16,000 *g* 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 aprotinin, 1 μmol/mL 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 X100 with 60 μg/mL aprotinin, and 2 μmol/mL PMSF) was used in this homogenization. The membrane fraction was allowed to sit on ice for 2 hr, then centrifuged for 10 min at 16,000 *g* 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 s in a microcentrifuge (Eppendorf) at 16,000 *g* 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 heated to 80° for 13 min to denature proteins, centrifuged (in an Eppendorf microcentrifuge for 20 s at 16,000 *g*) to precipitate the beads, and 15 μL of the supernatant loaded onto a 12% polyacrylamide Tris–glycine 8 cm × 10 cm, 1 mm thick commercial precast gel (Invitrogen). The proteins were separated by gel 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, 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 to 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 [22].

2.6. Band visualization by chemiluminescence

Membrane 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 preflash according to the manufacturer's protocol with a Sensitize preflash unit (Amersham Pharmacia Biotech), membranes were allowed to expose ECL Hyperfilm (Amersham Pharmacia Biotech) in an autoradiography cassette for 15 s to 3 min. Exposure time was adjusted to maintain samples within the linear response range as established in

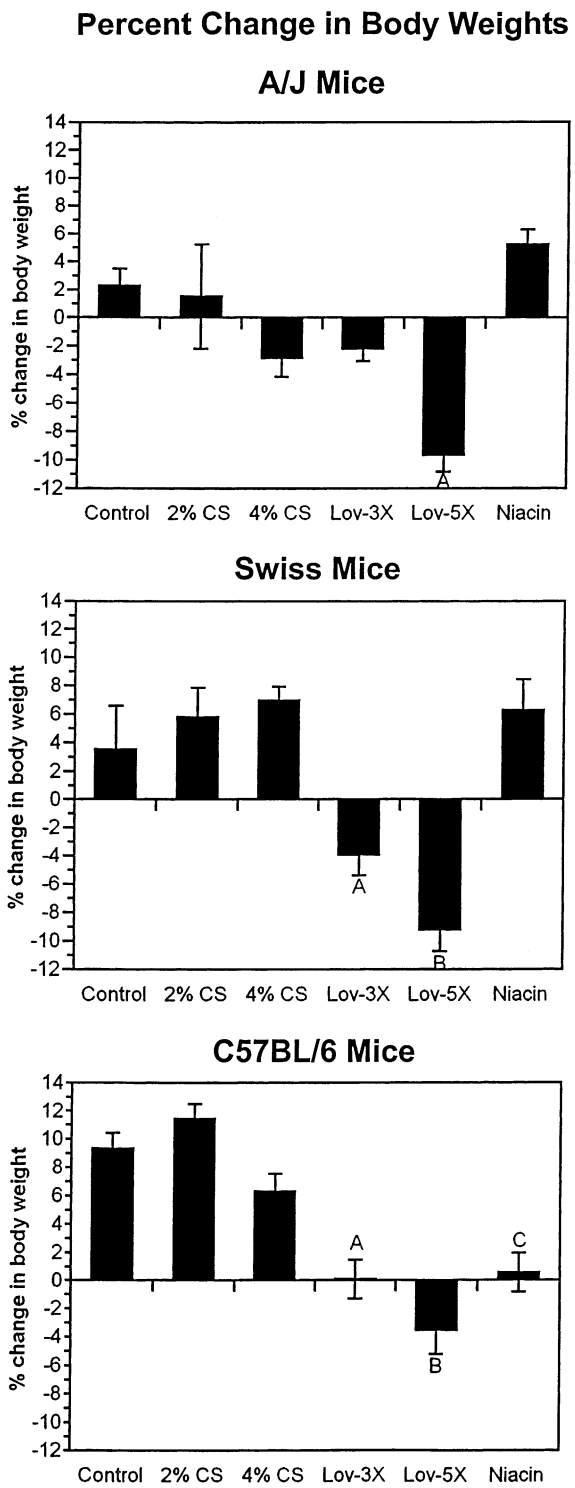

Fig. 1. Percent changes in body weight during the three-week experiment for female A/J, Swiss, and C57BL/6 mice. Treatments include control, 2 or 4% CS, Lov 25 mg/kg by intraperitoneal injection three (Lov-3×) or five (Lov-5×) times per week, or 1% niacin. Values shown by bars are mean ± SEM. Each bar represents the mean of values from five animals. Statistically significant differences were detected by ANOVA (Swiss and C57BL/6 mice) or the Kruskal–Wallis test (A/J mice) followed by pairwise Tukey or Dunn testing. Mean initial weights of the various treatment and control groups of mice ranged from 19.3 to 21.1 g (A/J), from 22.8 to 24.6 g (Swiss) or from 18.9 to 19.8 g (C57BL/6). 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 control ($P < 0.05$) and niacin ($P < 0.001$) groups. Middle panel (Swiss mice): (A) differs significantly from the 2% CS ($P < 0.05$), 4% CS ($P < 0.01$) and niacin ($P < 0.001$) groups; and (B) differs significantly from the control ($P < 0.01$) and the 2% CS, 4% CS, and niacin groups ($P < 0.001$). Lower panel (C57BL/6 mice): (A) differs significantly from the control and 2% CS groups ($P < 0.001$) and the 4% CS group ($P < 0.05$); (B) differs significantly from the control, 2% CS and 4% CS groups ($P < 0.001$); and (C) differs significantly from the control ($P < 0.01$) and 2% CS ($P < 0.001$) groups.

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

2.8. Comparisons between groups

Total, membrane, or cytoplasmic K-ras protein were compared to control samples run on the same blot. Each treatment within a given mouse strain was compared to the same five control samples, which 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. Each ratio was thus dependent on two densitometric values from the same blot. Differences in exposure, completeness of transfer, or other incidental differences 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 densitometric values was performed using GraphPad InStat v. 3.05 (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 which 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. Changes in animal body weights

The experimental treatments had differing effects on weight gain. Weight changes are shown graphically (Fig. 1)

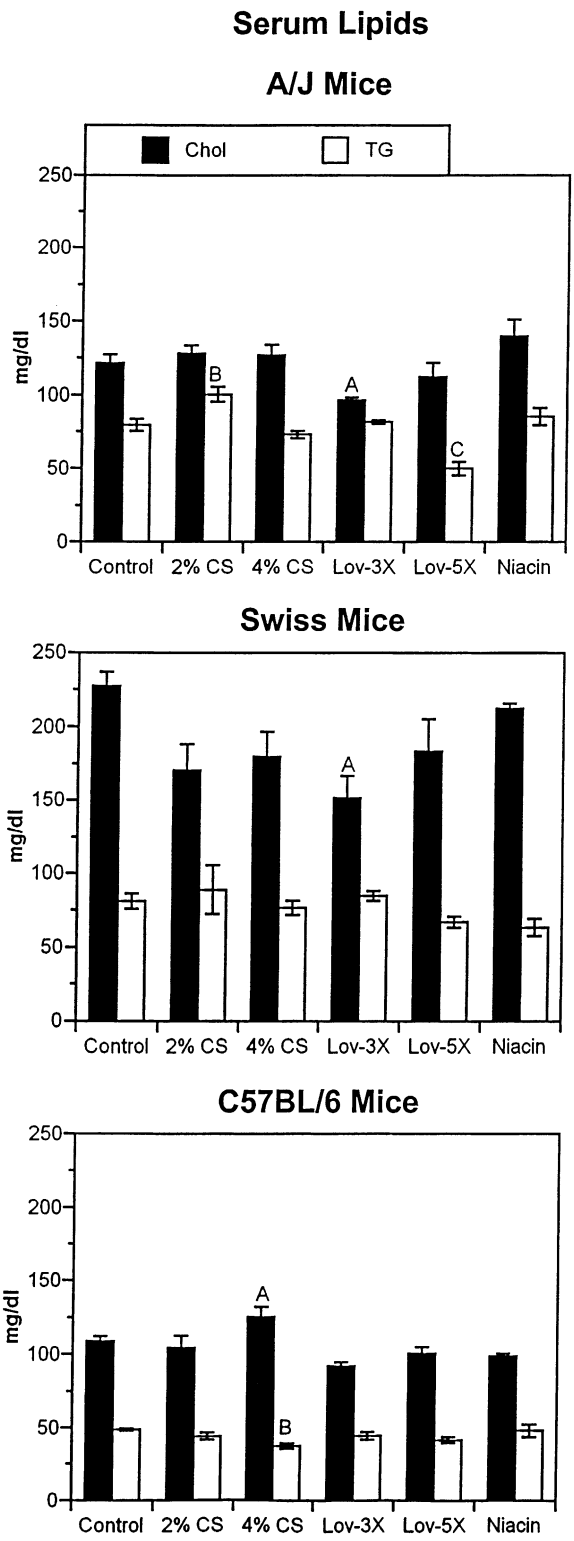

Fig. 2. Serum cholesterol and triglycerides in female A/J, Swiss, and C57BL/6 mice. Group identifications are the same as in Fig. 1. Values shown by bars are mean ± SEM. Each bar represents the mean of values from five animals. Upper panel (A/J mice): (A) cholesterol differs significantly from that of the niacin group ($P < 0.05$) only; (B) triglycerides differs significantly from those of the control and Lov-3× groups ($P < 0.05$), 4% CS ($P < 0.01$), Lov-5× ($P < 0.001$) groups; and (C) triglycerides differ significantly from those of the control, 2% CS, Lov-3×, and niacin groups ($P < 0.001$) and that of the 4% CS group ($P < 0.01$). Middle panel (Swiss mice): (A) cholesterol differs significantly from that of the control group ($P < 0.05$). Lower panel (C57BL/6 mice): (A) cholesterol differs significantly from those of the Lov-3× and Lov-5× groups ($P < 0.01$) and that of the niacin group ($P < 0.05$); and (B) triglycerides differ significantly from that of the control group ($P < 0.05$).

to show more clearly differences between groups and mouse strains. The percent changes shown were calculated by subtracting the initial body weight from the final weight, dividing this difference by the initial weight, and converting to a percentage. In general, changes in body weights over the three-week experiment were dependent on mouse strain, with control, niacin, and CS treatments usually resulting in weight gains, and Lov treatments resulting in weight loss. A/J mice tended to gain weight more poorly than the other two strains. It was noted that all strains of mice given Lov-5× had a statistically significant weight loss compared to their control groups. Statistically significant suppression of weight gain was also noted in C57BL/6 mice treated with Lov-3× or fed niacin.

3.2. Serum lipids

Serum cholesterol and triglycerides for the three mouse strains are shown in Fig. 2. Serum cholesterol was significantly reduced only in Swiss mice treated with Lov-3×. None of the hypocholesterolemic treatments was successful in reducing cholesterol significantly below control levels in the other mouse strains, although Lov-3× did produce nonsignificant reductions in cholesterol in A/J and C57BL/6 mice. In C57BL/6 mice, 4% CS treatment produced a serum cholesterol significantly higher than that of the Lov groups or niacin. Serum triglycerides were generally not altered compared to control. Exceptions were in A/J mice fed 2% CS, where triglycerides were significantly increased, or A/J mice treated with Lov-5×, where triglycerides were significantly decreased compared to control. Also, C57BL/6 mice fed 4% CS had a significantly decreased triglyceride value compared to control. Otherwise, no effects on triglycerides were statistically significant.

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 cytoplasm ratios are also shown as a percent of the control membrane to cytoplasm ratio for each mouse strain. Once again, results were mouse strain-dependent, but some overall patterns were observed. A/J mice had the largest number of significant treatment effects, while none of the treatments produced significant alterations in K-ras protein in Swiss mice. CS feeding effectively increased membrane K-ras protein levels in both A/J and C57BL/6 mice. This increase was also reflected by increased levels of total

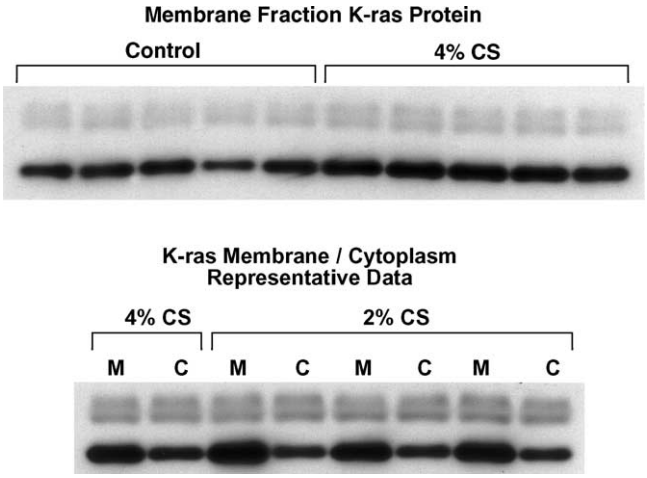

Fig. 3. Upper panel: representative data of a Western blot of immunoprecipitated K-ras protein in the membrane fractions of lung tissue lysates. 4% CS = 4% cholestyramine diet. The fainter upper bands are the mouse immunoglobulin light chains. Lower panel: representative Western blot of immunoprecipitated 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 and 4% CS = 4% cholestyramine diet. The upper bands are the mouse immunoglobulin light chains. 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.

K-ras protein, although these were not statistically significant in every case. Lov treatment increased total K-ras in both A/J and C57BL/6 mice. In A/J mice, increased levels of both membrane and cytoplasmic K-ras accompanied this increase. Unexpectedly, no change in either membrane or cytoplasmic K-ras could be detected in C57BL/6 mice treated with Lov. Niacin failed to produce any significant alterations in K-ras protein quantity or distribution in any of the three mouse strains.

4. Discussion

The original hypothesis underlying these experiments was substantiated only partially. First, it is clear that there is no relationship between actual reduction of cholesterol by a treatment and the alterations in K-ras protein observed. Only Lov-3×-treated Swiss mice had a significant reduction in serum cholesterol compared to control, but these mice had no significant alterations in K-ras protein in lung. Conversely, both CS and Lov in the other mouse strains produced alterations in K-ras protein, but serum cholesterol was unaffected. In two of the three mouse strains, CS treatment increased membrane K-ras protein as predicted. Such an increase might enhance tumorigenesis if K-ras were mutated. Indeed, CS has been found to be a tumor promotor in both mammary [15] and colon carcinogenesis [16] experiments. Lov did not have the hypothesized effect of reducing membrane K-ras. Indeed, there was actually an increase in membrane K-ras noted in A/J mice. As expected, niacin had no effect on K-ras protein.

The marked mouse strain differences observed in this study are of interest in view of the large differences apparent among humans in their susceptibility to lung cancer. Differences in cholesterol metabolism between mouse strains may be a partial explanation of our findings. For example, mice have been shown to have strain-dependent differences in susceptibility to diet-induced atherogenesis [23]. Serum cholesterol levels have been found to vary significantly among rat strains [24]. Significant strain differences in basal HMG-CoA reductase activity may be a partial explanation of the differences in effects on K-ras protein we observed between mouse strains. Hepatic HMG-CoA reductase levels have been documented to vary 24-fold among mouse strains [25]. It is not known if HMG-CoA reductase levels in lung also follow this pattern. It is also extremely difficult to down-regulate HMG-CoA reductase effectively in mice, due to compensatory dramatic increases in *de novo* synthesis of HMG-CoA reductase enzyme levels and activity in response to inhibition by HMG-CoA reductase inhibitor-type drugs [26,27]. This difficulty may explain why Lov failed to reduce membrane-associated K-ras quantity in any of the three strains of female mice studied. Our starting hypothesis, while logically attractive, now appears to be overly simplistic in predicting how the hypocholesterolemic agents might affect K-ras protein in lung.

None of the hypocholesterolemic agents was consistently effective in reducing cholesterol in our female mice. Only in one case (Lov-3× in Swiss mice) was cholesterol significantly reduced. The majority of published studies of niacin, Lov, and CS in mice also show a lack of significant lowering of serum cholesterol. Agent-specific hypotheses for the lack of effect of these hypocholesterolemics in mice have been advanced, which may explain the lack of effect in our study. Lov may be ineffective in reducing cholesterol in most cases because it is extremely difficult to down-regulate HMG-CoA reductase effectively in mice, due to dramatic increases in *de novo* synthesis of HMG-CoA reductase enzyme levels and activity in mice treated with Lov-type drugs [26,27]. Niacin generally reduces VLDL- and LDL-cholesterol in humans. Possibly it is ineffective in mice because in general mice have a low level of LDL-cholesterol with a high level of HDL [28], opposite of the typical human situation. CS studies have been done primarily in rats, although a recently published study found no change in serum cholesterol with 5% CS feeding in mice [29]. In rat studies, CS has been shown to decrease LDL-cholesterol while simultaneously increasing HDL, resulting in no change or even an increase in serum total cholesterol [13,15]. We suspect this same effect explains the lack of hypocholesterolemic effect for CS in mice.

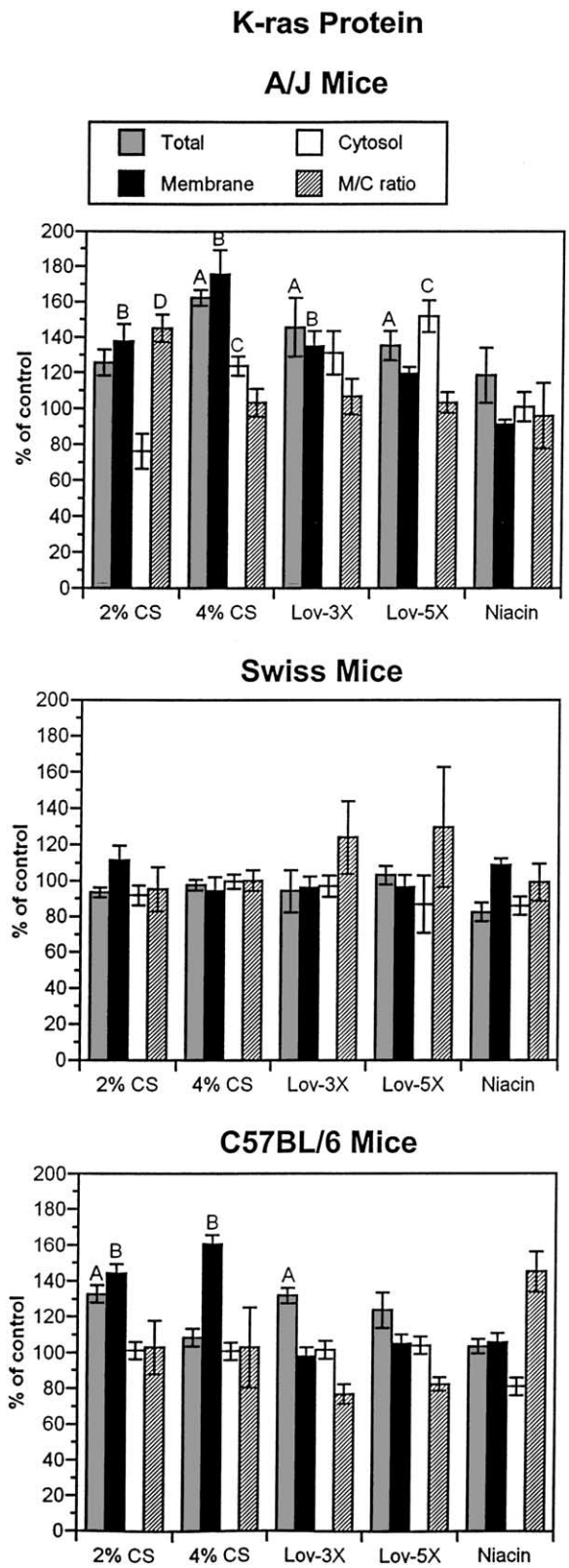

Fig. 4. Graphical representation of total, membrane, and cytoplasmic K-ras protein and K-ras protein membrane to cytoplasm ratios, expressed as a percent of control. Bars are mean ± SEM. Group identifications are the same as in Fig. 1. Each bar represents the mean of values from five animals, except as follows, where N = 4: A/J mice: total K-ras in the 2% CS group; Swiss mice: membrane K-ras in 4% CS and Lov-3× groups, and membrane to cytoplasm ratio in the 4% CS group; C57BL/6 mice: total K-ras in the 4% CS group. Control group membrane to cytoplasm ratios (mean ± SEM) were 8.7 ± 0.8 (A/J mice), 10.5 ± 1.1 (Swiss mice) and 10.7 ± 2.2 (C57BL/6 mice). Explanation of symbols above bars: upper panel (A/J mice): (A) total K-ras protein differs significantly from control, $P < 0.0001$ (for 4% CS) or $P < 0.05$ (for Lov-3× and Lov-5×); (B) membrane K-ras protein differs significantly from control $P < 0.005$ (4% CS) or $P < 0.05$ (2% CS and Lov-3×); (C) cytoplasmic K-ras protein differs significantly from control $P < 0.05$ (4% CS) or $P < 0.005$ (Lov-5×); and (D) membrane to cytoplasm ratio differs significantly from niacin group, $P < 0.05$. Middle panel (Swiss mice): no differences shown are statistically significant. Lower panel (C57BL/6 mice): (A): total K-ras protein differs significantly from control, $P < 0.05$ (2% CS) or $P < 0.01$ (Lov-3×); and (B): membrane K-ras protein differs significantly from control $P < 0.005$ (2% CS) or $P < 0.01$ (4% CS).

In this experiment, body weight gain varied considerably between treatments. Consideration should be given to whether this might have influenced the K-ras protein results. Comparison of the figure showing percent change in weight (Fig. 1) with that showing total, membrane, and cytoplasmic K-ras protein (Fig. 4) does not suggest any obvious relationship between the two for these female mice. A/J mice had a significant reduction in body weight gain in the Lov-5× group, but their pattern of alterations of K-ras protein did not differ greatly from that of the Lov-3× group, whose percentage weight change did not differ significantly from control. Swiss mice gained significantly less weight in the Lov groups compared to control, but none of the treatments in these mice resulted in significant effects on K-ras protein. Likewise, C57BL/6 mice had a significantly lower mean weight gain in both Lov groups and the niacin group compared to control, but there was no consistent pattern in changes in total, membrane, or cytoplasmic K-ras protein quantities in these groups. The Lov-3× group had an increase in total K-ras, but so did the 2% CS group which had a large weight gain rather than a weight loss. Therefore, there appears to be no consistent pattern of K-ras changes accompanying weight loss in any of the three mouse strains, and we do not consider it likely that our results are due to differences in body weight gains between treatment groups.

It has been suggested in the scientific literature that women may be more susceptible to tobacco-induced lung cancer than men [11,30], as well as having differences in response to dietary interventions aimed at lung cancer risk reduction [11,12]. It is therefore of interest to compare the results of the present study in female mice with those of our previous study performed in male mice [9]. The serum lipid results are contrasted in Fig. 5. In general, female mice tended to have a lower serum cholesterol than their male counterparts. Triglycerides tended to follow a similar trend, with generally slightly lower values in female mice. There was no sex difference in the effects of CS in any of the three mouse strains. It had no effect on cholesterol, increased triglycerides (2% CS only) in both sexes of A/J mice, and decreased triglycerides (4% CS only) in both sexes of C57BL/6 mice. Lov had sex-specific effects in A/J mice, where it significantly decreased triglycerides (Lov-5× only) in females, but increased triglycerides in males

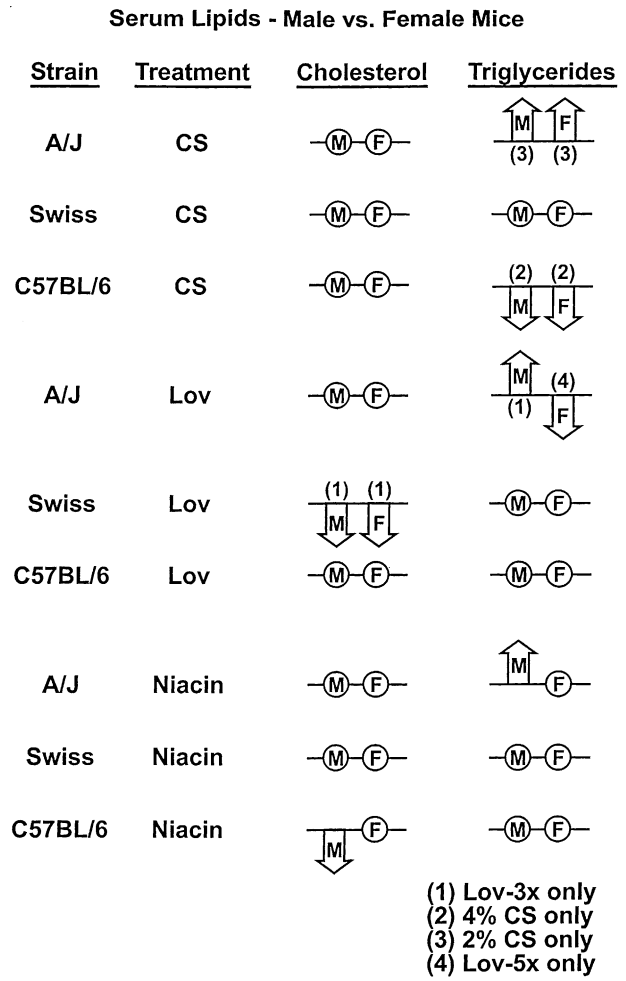

Fig. 5. Changes in serum cholesterol and triglycerides with each of the hypocholesterolemic agents, comparing results in male [9] and female mice (present study). Upward arrows indicate a significant increase relative to control, downward arrows a decrease, and a circle no change. M within a symbol is for male mice and F for female mice. Explanation of numbers shown below some of the arrows: (1) change occurred in Lov-3× group only, (2) change occurred in 4% CS group only, (3) change occurred in 2% CS group only, and (4) change occurred in Lov-5× group only.

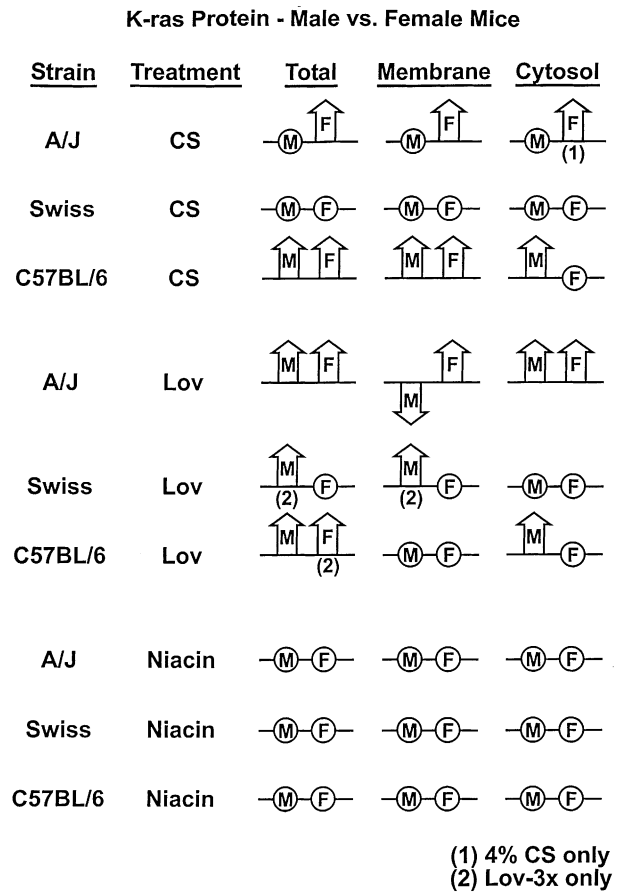

Fig. 6. Changes in K-ras protein with each of the hypocholesterolemic agents, comparing results in male [9] and female mice (present study). Upward arrows indicate an increase relative to control, downward arrows a decrease, and a circle no change. M within a symbol is for male mice and F for female mice. For the purposes of discussion and simplification, increases in membrane or cytoplasmic K-ras were considered to be significant only if they resulted in a significant increase in total K-ras. In four cases, all among the male mice, there were statistically significant differences in membrane or cytoplasmic K-ras protein without a significant increase in total K-ras. In each case, the resultant change in membrane or cytoplasmic K-ras protein was less than 30%, and these results are recorded as "no change" in the table. Explanation of numbers shown below some of the arrows: (1) change occurred in 4% CS group only and (2) change occurred in Lov-3× group only.

(Lov-3× only). In Swiss and C57BL/6 mice, Lov treatment had similar effects in both sexes. Niacin in most cases had no effect on serum cholesterol or triglycerides in either sex in any of the three mouse strains. Niacin, however, did increase triglycerides in male A/J mice and decrease cholesterol in male C57BL/6 mice.

Fig. 6 compares the lung K-ras protein levels of male and female mice of the three strains for each of the hypocholesterolemic agents. For the purposes of discussion and simplification, increases in membrane or cytoplasmic K-ras were considered to be significant only if they resulted in a significant increase in total K-ras (see figure legend). Sex-dependent differences were especially prominent in A/J mice. Only female A/J mice responded to CS with increases in total, membrane, and cytoplasmic K-ras, while Lov treatment resulted in an increase in membrane K-ras in females, but a decrease in males. In Swiss mice, on the other hand, Lov caused an increase in total and membrane K-ras in males, but no change in females. In C57BL/6 mice, effects of the hypocholesterolemic agents on K-ras protein levels were most often the same in males and females.

The increase in membrane-associated K-ras observed in A/J females treated with CS or Lov compared to males may have important implications in the process of lung carcinogenesis. If one considers that increased K-ras protein within the membrane fraction might be a liability if K-*ras* were to become mutated, female A/J mice fed CS would have an enhanced lung cancer risk compared to males, and Lov would be less protective in female mice than in males. A published lung carcinogenesis chemoprevention study fed Lov to male A/J mice and found a modest (but still

statistically significant) protective effect [17]. It would be of interest to see whether a protective effect would also be observed if this experiment were conducted in female A/J mice.

The effect of CS or Lov on K-ras protein differed in the female mice in our study in eight instances compared to the published results in male mice [9] (Fig. 6). Possible mechanisms might include sex-related differences in drug metabolism or the effects of estrogen on K-ras localization or expression levels. Sex-related differences in drug metabolism may have occurred with Lov in our mice. It has been reported that women have a 20–50% higher blood level of Lov compared to men after chronic dosing [31]. There is also indirect evidence of sex-related metabolic differences in mice. Asenjo-Barron *et al.* [32] found that Lov was considerably more toxic in male CD1 mice compared to females when fed at a 1% level in conjunction with a hypercholesterolemic diet. Sex differences in ras expression has been reported in other published studies in response to treatment with toxins or chemotherapeutic drugs. Sex-related differences in ras expression have been documented in adipose tissue of guinea pigs treated with 2,3,7,8-tetracholrodibenzo-*p*-dioxin (TCCD) [33]. In another study [34], male and female mice treated with combination chemotherapy had large differences in the levels of immediate and delayed H-ras expression in lymphatic tissues. Estrogen as a mediator of sex differences in ras response may also be a plausible mechanism for our results. Estrogen receptors (both α and β) have been found in the lung of both mouse [35] and humans [36]. Treatment of various types of cultured cells with estrogen results in activation of MAP kinase signaling by increasing levels of GTP-bound ras protein (as reviewed in [37]). GTP-bound ras is translocated to the cell membrane, and this may have resulted in some of the increases in membrane bound K-ras protein seen in females but not males (e.g. Fig. 6, A/J mice) in our study. The overall mechanism is likely complex, perhaps involving both sex- and strain-specific differences in metabolism of Lov as well as estrogenic effects. Other mechanisms not described in the literature also remain a possibility.

In the past two years, a number of clinical and epidemiological studies have been published examining the effect of HMG-CoA reductase inhibitors on cancer development during long-term use. These studies were conducted in part because of persistent concerns raised regarding increased cancer deaths in treated patients in early lipid-lowering trials using CS or fibric-acid derivatives. Another motivation was to examine whether cancer cell inhibitory properties of the HMG-CoA reductase inhibitors observed *in vitro* would translate to decreased cancer incidence in patients treated with these drugs. These studies [38–43] are basically in agreement that the HMG-CoA reductase inhibitors appear to have no effect, either protective or deleterious, on cancer development for median periods of use of two [43] or five to seven years [38–42]. One study suggested a statistically nonsignificant protective effect ($P = 0.08$) for simvastatin [38], but this was not found in the other studies. What might explain the apparent lack of a protective effect? The dose used clinically to reduce cholesterol is, of course, much lower than that which was used in animal and *in vitro* studies. It is possible that the duration of these studies was not long enough to show a protective effect. As we found in mice, it is possible that only people with a particular genetic make up respond in a favorable manner to the HMG-CoA reductase inhibitors from a cancer prevention perspective, and that the human studies would need to be much larger or study only certain human genotypes to document an effect. It is possible that only certain types of cancer (such as ras-dependent cancers) may be affected, and that insufficient numbers of these were found in the study groups to show an effect. Lastly, it is possible these drugs simply are not protective in humans. Given the widespread and ever-increasing use of these drugs, it is nonetheless reassuring that at least they probably do not promote cancer.

Our experiments have involved treatment of mice with agents which may tend to promote (e.g. CS) or inhibit (e.g. Lov) lung tumorigenesis, at least in certain strains of mice. We observed a sex difference in response to these agents in A/J mice. Sex differences in response to tumor promoters have been reported in the literature, for example, in hepatic carcinogenesis [44] as well as in skin carcinogenesis [45]. Similarly, sex differences have been reported in response to chemopreventive agents, such as in a study of lycopene in chemoprevention of carcinogen-induced lung tumors, where it was effective in male B6C3F1 mice but had no effect in females [46]. Examination of the published studies cited in a recent review of animal experiments in lung cancer chemoprevention [47] suggests that all but two studies used only one sex of animals to reach their conclusions. One of these two was a study of the effect of α-napthoflavone protection against methylcholanthrene-induced lung tumors in male and female progeny of mouse dams treated with these compounds during gestation. Results showed little difference in response between male and female offspring [48]. The other study that used both sexes of mice [49] assayed multiple putative chemoprotective agents, but did not examine sex differences statistically. Some differences were apparent by inspection, however, especially in the effects of 13-cis retinoic acid (protective in males, tumor promoting in females). Our results and those cited above make a good case for studying potential chemopreventive or promotional agents in both sexes in order to reach a generally applicable conclusion.

Our results clearly show that hypocholesterolemic agents used clinically can dramatically alter K-ras protein quantity and distribution in lung in female mice. Strain-dependent differences suggest that such effects, if they were to occur in humans, would be highly dependent on the individual genetic makeup of a particular person. The sex-related differences we detected in response to the

hypocholesterolemic agents suggest that in future trials of diet or chemopreventive strategies aimed at reducing lung cancer, it would be important to include large numbers of both men and women as subjects.

Acknowledgments

This work was supported by the US Department of Health and Human Services and a Research Career Award NIH-HL00734 to David Kritchevsky. This publication has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract number N01-CD-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US government.

References

[1] Wingo PA, Ries LA, Giovino GA, Miller DS, Rosenberg HM, Shopland DR, Thun MJ, Edwards BK. Annual report to the nation on the status of cancer, 1973–1996, with a special section on lung cancer and tobacco smoking. J Natl Cancer Inst 1999;91:675–90.

[2] Kritchevsky SB, Kritchevsky D. Serum cholesterol and cancer risk: an epidemiologic perspective. Ann Rev Nutr 1992;12:391–416.

[3] Thrane EV, Becher R, Lag M, Refsnes M, Huitfeldt HS, Schwarze PE. Differential distribution and increased levels of ras proteins during lung development. Exp Lung Res 1997;23:35–49.

[4] Kammouni W, Ramakrishna G, Sithanandam S, Smith GT, Fornwald LW, Masuda A, Takahashi T, Anderson LM. Increased K-ras protein and activity in mouse and human lung epithelial cells at confluence. Cell Growth Diff 2002, in press.

[5] Zhang Z, Wang Y, Vikis HG, Johnson L, Liu G, Li J, Anderson MW, Sills RC, Hong HL, Devereux TR, Jacks T, Guan KL, You M. Wildtype K-ras2 can inhibit lung carcinogenesis in mice. Nat Genet 2001;29:25–33.

[6] Ramakrishna G, Sithanandam G, Cheng RYS, Fornwald LW, Smith GT, Diwan BA, Anderson LM. K-ras p21 expression and activity in lung and lung tumors. Exp Lung Res 2000;26:659–71.

[7] Ramakrishna G, Perella C, Birely L, Diwan BA, Fornwald L, Anderson L. Decrease in K-ras p21 and increase in Raf1 and activated Erk1 and 2 in murine lung tumors initiated by *N*-nitrosodimethylamine and promoted by TCDD. Toxicol Appl Pharmacol 2002;179:21–39.

[8] Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ. Isoprenoid addition to ras protein is the critical modification for its membrane association and transforming activity. Proc Natl Acad Sci USA 1992;89:6403–7.

[9] Calvert RJ, Ramakrishna G, Tepper S, Diwan BA, Anderson LM, Kritchevsky D. 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. Biochem Pharmacol 2002;64:41–8.

[10] Shriver SP, Bordeau HA, Gubish CT, Tirpak DL, Davis ALG, Luketich JD, Siegfried JM. Sex-specific expression of gastrin-releasing peptide receptor: relationship to smoking history and risk of lung cancer. J Natl Cancer Inst 2000;92:24–33.

[11] Payne S. 'Smoke like a man, die like a man'?: a review of the relationship between gender, sex and lung cancer. Soc Sci Med 2001;53:1067–80.

[12] Dorgan JF, Ziegler RG, Shoenberg JB, Hartge P, McAdams MJ, Falk RT, Wilcox HB, Shaw GL. Race and sex differences in associations of vegetables, fruits, and carotenoids with lung cancer risk in New Jersey (United States). Cancer Causes Control 1993;4:273–81.

[13] Kazanecki ME, Melhem MF, Spichty KJ, Kelly RH, Rao KN. Diet-induced reduction in serum lipoproteins stimulates cell proliferation in weanling rats. Pharmacol Res 1989;21:533–47.

[14] Turley SD, West CE. Effect of cholesterol and cholestyramine feeding and of fasting on sterol synthesis in liver, ileum, and lung of the guinea pig. Lipids 1976;11:571–7.

[15] Melhem MF, Gabriel HF, Eskander ED, Rao KN. Cholestyramine promotes 7,12-dimethylbenzanthracene induced mammary cancer in Wistar rats. Br J Cancer 1987;56:45–8.

[16] Nigro ND, Bhadrachari N, Chonchai C. A rat model for studying colon cancer: effect of cholestyramine on induced tumors. Dis Colon Rectum 1973;16:438–43.

[17] Hawk MA, Cesen KT, Siglin JC, Stoner GD, Ruch RJ. Inhibition of lung tumor growth and mouse lung tumor formation by lovastatin. Cancer Lett 1996;109:217–22.

[18] Narisawa T, Morotomi M, Fukaura Y, Hasebe M, Ito M, Aizawa R. Chemoprevention by Pravastatin, a 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor of *N*-methyl-*N*-nitrosourea-induced colon carcinogenesis in F344 rats. Jpn J Cancer Res 1996;87:798–804.

[19] Grundy SM, Mok HYI, Zech L, Berman M. Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. J Lipid Res 1981;22:24–36.

[20] Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939–51.

[21] Khan SG, Saxena R, Bickers DR, Mukhtar H, Agarwal R. Inhibition of ras p21 membrane localization and modulation of protein kinase C isoenzyme expression during regression of chemical carcinogen-induced murine skin tumors by lovastatin. Mol Carcinog 1995;12:205–12.

[22] Ramakrishna G, Anderson LM. Levels and membrane localization of the c-K-ras p21 protein in lungs of mice of different genetic strains and effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and Aroclor 1254. Carcinogenesis 1998;19:463–70.

[23] Paigen B, Morrow A, Brandon C, Mitchell D, Holmes P. Variation in susceptibility to atherosclerosis among inbred strains of mice. Atherosclerosis 1985;57:65–73.

[24] Kritchevsky D, Tepper SA. Serum cholesterol levels of inbred rats. Am J Physiol 1964;207:631–3.

[25] Hwa JJ, Zollman S, Warden CH, Taylor BA, Edwards PA, Fogelman AM, Lusis AJ. Genetic and dietary interactions in the regulation of HMG-CoA reductase gene expression. J Lipid Res 1992;33:711–24.

[26] Kita T, Brown MS, Goldstein JL. Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. J Clin Invest 1980;66:1094–100.

[27] Feingold KR, Mao-Qiang M, Proksch E, Menon GK, Brown BE, Elias PM. The lovastatin-treated rodent: a new model of barrier disruption and epidermal hyperplasia. J Invest Dermatol 1991;96:201–9.

[28] Gustafsson K, Kiessling H. The mouse as a model for evaluation of hypotriglyceridemic drugs effects of nicotinic acid, clofibrate, and ethyl 2-(4-dibenxofuranyloxyl)-2-methylpropionate on plasma lipoproteins. Z Versuchstierk 1982;24:271–7.

[29] Chen JY, Levy-Wilson B, Goodart S, Cooper AD. Mice expressing the human *CYP7A1* gene in the mouse CYP7A1 knock-out background lack induction of CYP7A1 expression by cholesterol feeding and have increased hypercholesterolemia when fed a high fat diet. J Biol Chem 2002;277:42588–95.

[30] Zang EA, Wynder EL. Differences in lung cancer risk between men and women: examination of the evidence. J Natl Cancer Inst 1996;88:183–92.

[31] Cheng H, Rogers JD, Sweany AE, Dobrinska MR, Stein EA, Amin RD, Quan H. Influence of age and gender on the plasma profiles of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitory activity following multiple doses of lovastatin and simvastatin. Pharm Res 1992;9:1629–33.
[32] Asenjo-Barron JC, Cardenas-Vazquez R, Martinez F, Jaurez-Oropeza MA, Diaz-Zagoya JC. High lovastatin doses combined with hypercholesterolemic diet induce hepatic damage and are lethal to the CD-1 mouse. Life Sci 1999;64:2155–61.
[33] Enan E, El-Sabeawy F, Overstreet J, Matsumura F, Lasley B. Mechanisms of gender-specific TCDD-induced toxicity in guinea pig adipose tissue. Reprod Toxicol 1998;12:357–69.
[34] Ember I, Kiss I, Ghodratollah N, Raposa T. Effect of ABVD therapeutic protocol on oncogene and tumor suppressor gene expression in CBA/Ca mice. Anticancer Res 1998;18:1149–52.
[35] Couse JH, Lindzey J, Grandein K, Gustafsson J-A, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-α (Erα) and estrogen receptor-β (Erβ) messenger ribonucleic acid in the wild type and Erα-knockout mouse. Endocrinology 1997;138:4613–21.
[36] Stabile LP, Davis ALG, Gubish CT, Hopkins TM, Luketich JD, Christie N, Finkelstein S, Siegfried JM. Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor α and β and show biological responses to estrogen. Cancer Res 2002;62:2141–50.
[37] Stanten RJ, Song RX, McPherson R, Kumar R, Adam L, Jeng M-H, Yue W. The role of mitogen-activated protein (MAP) kinase in breast cancer. J Steroid Biochem Molec Biol 2002;80:239–56.
[38] Pedersen TR, Wilhemnsen L, Faergeman O, Starndberg TE, Thorgeirsson G, Troedsson L, Kristianson J, Berg K, Cook TJ, Haghfelt T, Kjekshus J, Miettinen T, Olsson AG, Pyorala K, Wedel H. Follow-up study of patients randomized in the Scandinavian simvastatin survival study (4S) of cholesterol lowering. Am J Cardiol 2000;86: 257–62.
[39] Pfeffer MA, Keech A, Sacks FM, Cobbe SM, Tonkin A, Byington RP, Davis BR, Friedman CP, Braunwald E. Safety and tolerability of pravastatin in long-term clinical trials—prospective pravastatin pooling project. Circulation 2002;105:2341–6.
[40] Coogan PF, Rosenberg L, Palmer JR, Strom BL, Zauber AG, Shapiro S. Statin use and the risk of breast and prostate cancer. Epidemiology 2002;13:262–7.
[41] Downs JR, Clearfield M, Tyroler AH, Whitney EJ, Kruyer W, Langendorfer A, Zagrebelsky V, Weis S, Shapiro DR, Beere PA, Gotto AM. Air Force/Texas coronary atherosclerosis prevention study (AFCAPS/TexCAPS): additional perspectives on tolerability of long-term treatment with lovastatin. Am J Cardiol 2001;87:1074–9.
[42] Clearfield M, Downs JR, Weis S, Whitney EJ, Kruyer W, Shapiro DR, Stein EA, Langendorfer A, Beere PA, Gotto AM. Air Force/Texas coronary atherosclerosis prevention study (AFCAPS/TexCAPS): efficacy and tolerability of long-term treatment with lovastatin in women. J Womens Health Gend Based Med 2001;10:971–81.
[43] Kaye JA, Meier CR, Walker AM, Jick H. Statin use, hyperlipidaemia, and the risk of breast cancer. Br J Cancer 2002;86:1436–9.
[44] Blanck A, Hansson T, Gustafsson J-A, Eriksson LC. Pituitary grafts modify sex differences in liver tumor formation in the rat following initiation with diethylnitrosamine and different promotion regimens. Carcinogenesis 1986;7:981–5.
[45] Moser GJ, Robinette CL, Smart RC. Characterization of skin tumor promotion by Mirex: structure–activity relationships, sexual dimorphism and presence of Ha-ras mutation. Carcinogenesis 1993;14:1155–60.
[46] Kim DJ, Takusuka N, Nishino H, Tsuda H. Chemoprevention of lung cancer by lycopene. Biofactors 2000;13:95–102.
[47] You M, Bergman G. Preclinical and clinical models of lung cancer chemprevention. Hematol Oncol Clin North Am 1998;12:1037–51.
[48] Anderson LM, Priest LJ. Reduction in the transplacental carcinogenic effect of methylcholanthrene in mice by prior treatment with α-napthoflavone. Res Commun Chem Pathol Pharmacol 1980;30: 431–45.
[49] Yun T-K, Kim S-H, Lee Y-S. Trial of a new medium-term model using benzo(a)pyrene induced lung tumor in newborn mice. Anticancer Res 1995;15:839–46.