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Elevated K-ras activity with cholestyramine and lovastatin, but not konjac mannan or niacin in lung—Importance of mouse strain

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ABSTRACT

Our previous work established that hypocholesterolemic agents altered K-ras intracellular localization in lung. Here, we examined K-ras activity to define further its potential importance in lung carcinogenesis. K-ras activity in lungs from male A/J, Swiss and C57BL/6 mice was examined. For 3 weeks, mice consumed either 2 or 4% cholestyramine (CS), 1% niacin, 5% konjac mannan (KM), or were injected with lovastatin 25 mg/kg three or five times weekly (Lov-3X and Lov-5X). A pair-fed (PF) group was fed the same quantity of diet consumed by the Lov-5X mice to control for lower body weights in Lov-5X mice. After 3 weeks, serum cholesterol was assayed with a commercial kit. Activated K-ras protein from lung was affinity precipitated with a Raf-1 ras binding domain-glutathione-S-transferase fusion protein bound to glutathione-agarose beads, followed by Western blotting, K-ras antibody treatment, and chemiluminescent detection. Only KM reduced serum cholesterol (in two of three mouse strains). In C56BL/6 mice treated with Lov-3X, lung K-ras activity increased 1.8-fold versus control ($p = 0.009$). In normal lung with wild-type K-ras, this would be expected to be associated with maintenance of differentiation. In A/J mice fed 4% CS, K-ras activity increased 2.1-fold ($p = 0.02$), which might be responsible for the reported enhancement of carcinogenesis in carcinogen-treated rats fed CS. KM feeding and PF treatment had no significant effects on K-ras activity. These data are consistent with the concept that K-ras in lung has an oncogenic function when mutated, but may act as a tumor suppressor when wild-type.

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1. Introduction

It has been suggested that excess cancer deaths observed in the lipid-lowering trials of the 1970s and early 1980s might relate to the use of bile acid binding resins such as cholestyramine (CS) in many of these trials [1]. The *K-ras* gene is often mutated in mouse and human lung cancer, and lipid moieties derived from cholesterol metabolism are needed to anchor K-ras in the cell membrane where it can become activated. We showed that the lipid lowering agents cholestyramine (CS), lovastatin (Lov), and niacin can alter the intracellular distribution of K-ras protein between the membrane and cytoplasmic compartments in mouse lung [2,3]. However, there generally were not consistent responses to these agents borne out in all three strains of mice studied, eventually causing us to consider whether K-ras activity state might be a better determinant of K-ras effects in lung.

As the K-ras subcellular distribution studies [2,3] were under way, evidence began to accumulate that K-ras could act as a lung tumor suppressor in mice [4]. K-ras activity was reported to increase in nontransformed mouse lung cells (E10) at confluence to levels much higher than in proliferating cells [5]. Further evidence was a finding of reduced K-ras activity in mouse lung tumors compared to uninvolved lung [6]. These lines of evidence suggested that the activity state of K-ras in lung is an important determinant of its effect on tumorigenesis. Considering some clinical [7–9] and animal [10–12] evidence that HMG-CoA reductase (hydroxymethylglutaryl-coenzyme A reductase, E.C. #1.1.1.88) inhibitor drugs (''statins'') may act as tumor inhibitors, we decided to study the effect of lovastatin on K-ras activity in mouse lung. CS (a tumor promoter in carcinogen-treated rats), niacin, and konjac mannan (a dietary fiber derived from a Japanese tuber shown to lower cholesterol [13]), each of which are hypocholesterolemic agents with different mechanisms of action, were studied for the purpose of comparing their effects on K-ras activity with Lov. These studies were intended to shed further light on how these agents may inhibit or promote lung carcinogenesis. We treated male A/J (lung carcinogen sensitive), Swiss (intermediate), and C57BL/6 mice (carcinogen resistant) with CS, Lov, niacin, and konjac mannan for 3 weeks and measured serum cholesterol and K-ras activity in lung tissue.

2. Materials and methods

2.1. Experimental animals and treatments

Male A/J, Swiss, and C57/BL6 mice were obtained at 8 weeks of age from the National Cancer Institute at Frederick Animal Facility breeding colony. After a 1-week adaptation period, they were divided into eight treatment groups, with five mice of each strain per group. The groups were control, 2% CS (Upshur Smith Laboratories, Inc., Minneapolis, MN, USA), 4% CS, lovastatin (Merck Inc., West Point, PA, USA) 25 mg/kg i.p. three times per week (Lov-3X), and lovastatin 25 mg/kg i.p. five times per week (Lov-5X), 1% niacin (Sigma, St. Louis, MO), 5% konjac mannan (KM, Propol A, Shimizu Chemical Company, Hiroshima, Japan), and Pair-fed (PF, fed to match the spontaneous food intake of the Lov-5X group). The PF group was as an additional control for the low weight gain or weight loss in the Lov-5X group we noted in our previous studies. The composition of the control diet [14] is shown in Table 1. CS, KM, or niacin were substituted for corn starch in their respective diets. Diets were prepared in pelleted form to our specifications by Dyets, Inc., Bethlehem, PA, USA. Lov doses were chosen based on a published study [15] using these doses to produce measurable changes in H-ras membrane association, and were the same as those used in our previous two studies [2,3]. 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 lovastatin were fed the control diet. Animals had free access to diets (except in the pair-fed group, where diet was rationed) and to acidified tap water throughout the experiment. Animals 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, serum separated and frozen. Lungs were removed and frozen intact in liquid nitrogen and stored at −80 °C until analysis.

2.2. Serum cholesterol analysis

Frozen sera were thawed and analyzed for total cholesterol concentration using a commercial enzymatic kit.

2.3. Lung tissue preparation

Lungs were homogenized in 25 mM Hepes, pH 7.5, 150 mM NaCl, 1% Nonidet P40 (ethylphenyl-polyethyleneglycol), 0.25% Na deoxycholate, 10% glycerol, 10 mM $MgCl_2$, and 1 mM EDTA. Proteinase inhibitors [2 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 1 mM Na orthovanadate and 0.044 TIU (trypsin inhibitor units)/ml aprotinin] were added just prior to use. All of the above were obtained from Sigma (St. Louis, MO, USA) except for glycerol (Invitrogen, Carlsbad, CA, USA) and Nonidet P40 (USB, Cleveland, OH, USA). Two ml of buffer was used for each 100 mg of lung tissue. Lungs were homogenized in a glass vessel on ice with a motor-driven

Table 1 – Composition of the control diet

Ingredient	Grams/kg
Casein	140
Cornstarch	465.692
Dextrose	155
Sucrose	100
Cellulose	50
Soybean Oil	40
t-Butylhydroquinone	0.008
Salt mix [14]	35
Vitamin mix [14]	10
L-Cystine	1.8
Choline bitartrate	2.5

Teflon pestle for 20 strokes. The homogenate was centrifuged at 300 × *g* in a Sorval centrifuge for 10 min at 4 °C to remove nuclei and large insoluble particles. The supernatant was centrifuged again at (16,000 × *g*) in an Eppendorf microfuge for 10 min at 4 °C to remove any remaining insoluble materials. The final supernatant was frozen in liquid nitrogen and stored at −80 °C for no longer than 30 days prior to analysis. Total protein content of lung homogenates was determined using the BCA kit (Pierce, Rockford, IL, USA).

2.4. Activated K-ras assay

The principle of the assay is exclusive binding of GTP-bound (activated) ras to raf1. GDP-bound (inactivated) ras does not bind to raf1. Lung homogenates were assayed for activated (GTP-bound) ras by affinity binding to the ras binding domain (RBD) of raf1 fused to a glutathione-S-transferase (GST) protein attached to glutathione agarose beads. This allowed activated K-ras to be separated from the non-bound (GDP form) K-ras by centrifugation. Care was taken to consistently use the GST-RBD beads 5–6 days after preparation. The method, included preparation of the GST-RBD beads, has been published previously in detail [16].

Briefly, lung homogenate containing 400–600 μg total protein as prepared above was used for each 600 μl assay tube. The homogenate was diluted with ice cold 25 mM Hepes, 150 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$ with 2 mM phenylmethylsulfonyl fluoride and 25 mM NaF (all from Sigma, St. Louis, MO, USA) to produce a total volume in each tube of approximately 500 μl. Thirty microliters of a slurry of the GST-RBD beads was added to each tube (on ice). The tubes were placed on a rotator in a 4 °C cold room for 2 h. The tubes were removed and centrifuged in an Eppendorf 5414 microcentrifuge at 16,000 × *g* for 20 s. The supernatant was removed to within about 3 mm of the top of the pellet (to avoid aspirating the GST-RBD beads). Fresh buffer was added, and the tubes mixed by inversion several times before repeat centrifugation. These steps were repeated twice more as above. After the third wash, all of the supernatant was carefully removed. The final 100 μl was removed with a round gel-loading tip, to help avoid aspirating the beads.

2.5. Gel electrophoresis and Western blotting

Gel electrophoresis and Western blotting were used to visualize the recovered activated K-ras protein as described in detail elsewhere [3]. Briefly, 30–35 μl of 2× Lamelli loading buffer [(100 mM Tris HCl, 0.002%, w/v bromphenol blue] (both from Sigma, St. Louis, MO, USA), 400 mM dithiothreitol (Promega, Madison, WI, USA), 20% (v/v) glycerol (Invitrogen Life Technologies, Carlsbad, CA, USA) 4% SDS (BioRad Laboratories, Hercules, CA, USA)] was added to each tube. Tubes were heated at 80 °C for 13 min. After centrifugation, samples were loaded on a 12% polyacrylamide tris–glycine gel (Invitrogen, Carlsbad, CA, USA) and run for 2 h at 120 V. The gel was then transferred to a nitrocellulose (Hybond ECL™ #RPN303D, GE Health Care, Piscataway, NJ, USA, formerly Amersham) membrane by electroblotting. The blot was treated with a primary K-ras antibody [anti-c-K-Ras (Ab-1) (mouse), #OP24S, Oncogene Sciences, Cambridge, MA, USA] and a secondary antibody [anti-mouse IgG horseradish peroxidase-linked whole antibody (#NA931V, GE Health Care UK, Little Chalfont, Buckinghamshire, UK). The blots were treated with ECL™ chemiluminescence reagent #RPN2106, and then exposed to ECL Hyperfilm™ #RPN3114K (both from Amersham Biosciences UK Limited, Little Chalfont, Buckinghamshire, UK). K-ras activity blots for each treatment group were run with the control samples for the appropriate mouse strain on the same blot, for a total of seven blots for each strain. Blots were quantified by densitometry [Molecular Dynamics Personal Densitometer equipped with Image Quant™ version 3.3 software (Molecular Dynamics, Sunnyvale, CA, USA)].

2.6. Statistical analysis

Statistical analysis was performed using GraphPad InStat™ v. 3.05 (GraphPad Software, http://www.graphpad.com/). 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. Linear regressions were performed as indicated to document proposed interactions of two experimental variables. To examine possible dose response effects for statistical significance, a Pearson correlation was performed with consultation from the Computer and Statistical Services department at the National Cancer Institute at Frederick. Differences were considered statistically significant when *p*-values were less than 0.05.

3. Results

3.1. Body weights

Body weight change as a percentage of the initial body weight is shown in Fig. 1. Weight gains for individual mice tended to be quite variable. Among all three mouse strains, most groups consistently showed weight gain, without statistically significant differences between groups. A notable exception were mice in the Lov-5X and the pair-fed groups. In Swiss mice weight gain was only 0.5 ± 1.9% (mean ± S.E.M., $p < 0.05$ versus KM group) and 2.6 ± 3.6% for Lov-5X and pair-fed mice respectively versus 15.1 ± 5.0% for the control group. In A/J and C57BL/6 mice there was actual weight loss. A/J mice had a mean loss of −5.4 ± 5.5% and −3.9 ± 2.7% for Lov-5X and pair-fed respectively, versus a 3.2 ± 1.5% weight gain for the control group. These differences in A/J mice were not statistically significant. C57BL/6 mice had mean weight losses of −2.1 ± 3.2% ($p < 0.01$ versus control) and −7.1 ± 3.3% ($p < 0.001$ versus control) for Lov-5X and pair-fed, respectively, compared to a mean weight gain for the control group of 14.4 ± 2.4%. These changes differed significantly from control only for the C57BL/6 mice as indicated in the figure. Lov-3X A/J mice also lost about 2% of initial weight (not statistically significant).

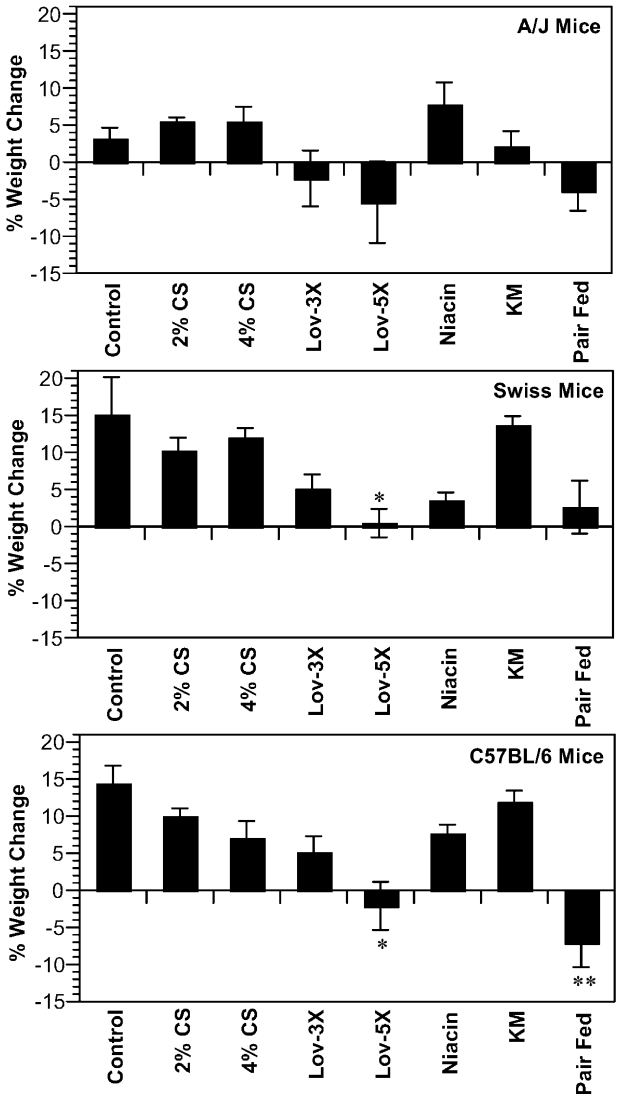

Fig. 1 – Percent weight change [((final weight − initial weight)/initial weight) × 100] in mice treated with hypocholesterolemic agents for 3 weeks. Treatment groups include control, 2 or 4% cholestyramine (CS), lovastatin 25 mg/kg i.p. three or five times per week (Lov-3X or Lov-5X), 1% niacin, 5% konjac mannan (KM) or pair-fed. Bars are mean ± S.E.M. Each bar represents the mean from five mice, except for $n = 4$ in the A/J mice in the Lov-5X and pair-fed groups. Statistical tests used were the Kruskal–Wallis test followed by Dunn's test (A/J mice), or 1-way ANOVA followed by the Tukey test (Swiss and C57BL/6 mice). AJ mice: no differences are statistically significant. Swiss mice: asterisk (*) denotes significantly different from KM group only ($p < 0.05$). C57BL/6 mice: asterisk (*) denotes significantly different from control ($p < 0.001$), 2% CS ($p < 0.05$) and KM ($p < 0.01$). **Significantly different from control ($p < 0.001$) and all other treatments except Lov-5X.

3.2. *Serum cholesterol*

Cholesterol was significantly decreased from control in only two instances, by KM in both Swiss (by 28 ± 4%, $p < 0.05$) and C57BL/6 mice (by 41 ± 3.3%, $p = 0.01$) as shown in Fig. 2. There were several other statistically significant differences among different groups of Swiss mice as shown by letters in the figure, but only KM feeding

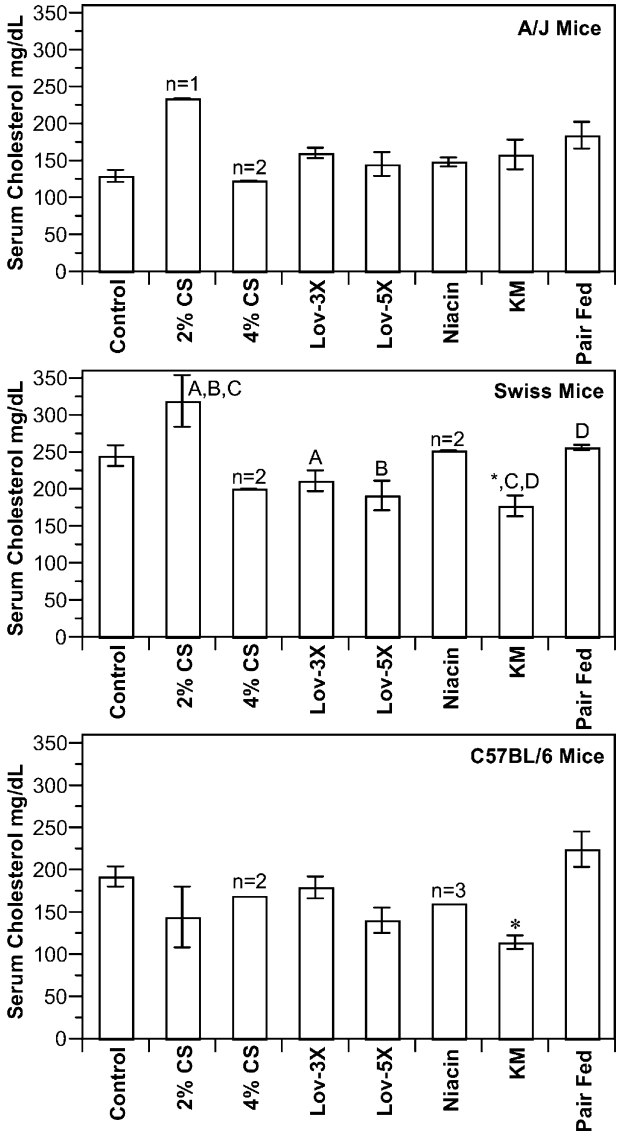

Fig. 2 – Serum cholesterol (mg/dl) in mice treated with hypocholesterolemic agents for 3 weeks. Treatment groups include control, 2 or 4% cholestyramine (CS), lovastatin 25 mg/kg i.p. three or five times per week (Lov-3X or Lov-5X), 1% niacin, 5% konjac mannan (KM) or pair-fed. Bars are mean ± S.E.M. Each bar represents the mean from 4 to 5 animals, except $n = 8$–10 for KM and control. Due to technical difficulties, for some treatment groups, sample size was 1–3, which is shown above the appropriate bar. Groups with only one or two cholesterol values were excluded from the statistical analysis by 1-way ANOVA followed by Tukey's test. A/J mice: no significant differences. Swiss mice: asterisk (*) denotes KM group had a significantly reduced cholesterol vs. control ($p < 0.05$). Columns sharing a letter differ significantly from each other ($p < 0.05$). C57BL/6 mice: asterisk (*) denotes KM group had a significantly reduced cholesterol vs. control ($p = 0.01$).

resulted in cholesterol levels that differed significantly from control.

3.3. Levels of activated K-ras protein

An example blot showing Lov-3X versus control from five C57BL/6 mice versus the five control mice for this strain is shown in Fig. 3. Blots of this sort were done for all seven treatments of each mouse strain, with each blot for a given strain containing the same strain-specific control specimens. Levels of activated K-ras protein are shown in Fig. 4 as a percentage of the activated K-ras level of the control group for the appropriate mouse strain. In general, there were few statistically significant alterations in activated K-ras levels between groups. Swiss mice had a smaller magnitude of change with the various treatments compared to the other strains. The only statistically significant alterations from control K-ras activity levels was with 4% CS in A/J mice (208 ± 27% of control, $p = 0.02$) and Lov-3X treatment in C56BL/6 mice (181 ± 16% of control, $p = 0.009$). Two percent CS also elevated activated K-ras levels in A/J mice to 163% of control, which was not itself statistically significant compared to control. However, when analyzed for dose response, a significant linear correlation was found between CS dose (2 or 4%) and activated K-ras protein level in A/J mice ($p < 0.001$, Pearson correlation).

3.4. Correlation of K-ras activity with body weight changes

One question raised in our previous studies was whether the effect of Lov might be due to animal weight loss compared to the other treatments. Correlating average weight gains versus average K-ras activity for all groups by mouse strain gave non-significant p-values between 0.23 and 0.66. To address this question more directly, a PF group, where each mouse was fed the same quantity of diet consumed by a Lov-5X partner mouse was included. We examined by linear regression whether a significant relationship might exist between K-ras activity and weight change in the pair-fed and Lov-5X animals. Individual animal body weight changes in the Lov-5X and pair-fed mice were plotted against their individual activated K-ras levels (expressed as a percent of the mean of the control samples run on the same blot). Linear regressions showed no significant correlations between change in body weight of individual mice and its respective K-ras activity level in any mouse strain, with

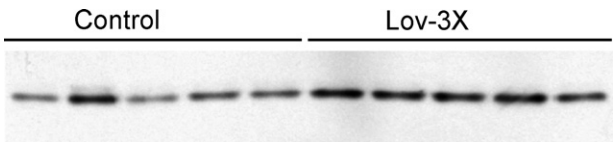

Fig. 3 – Representative data from K-ras activity Western blot. The result is from C57BL/6 mice. The Lov-3X group was compared on this blot with the control group for C57BL/6 mice. The mean densitometric value for Lov-3X was significantly higher than that of the control group ($p = 0.009$). Seven such blots, comparing a different treatment group to the control, were prepared for each of the three mouse strains.

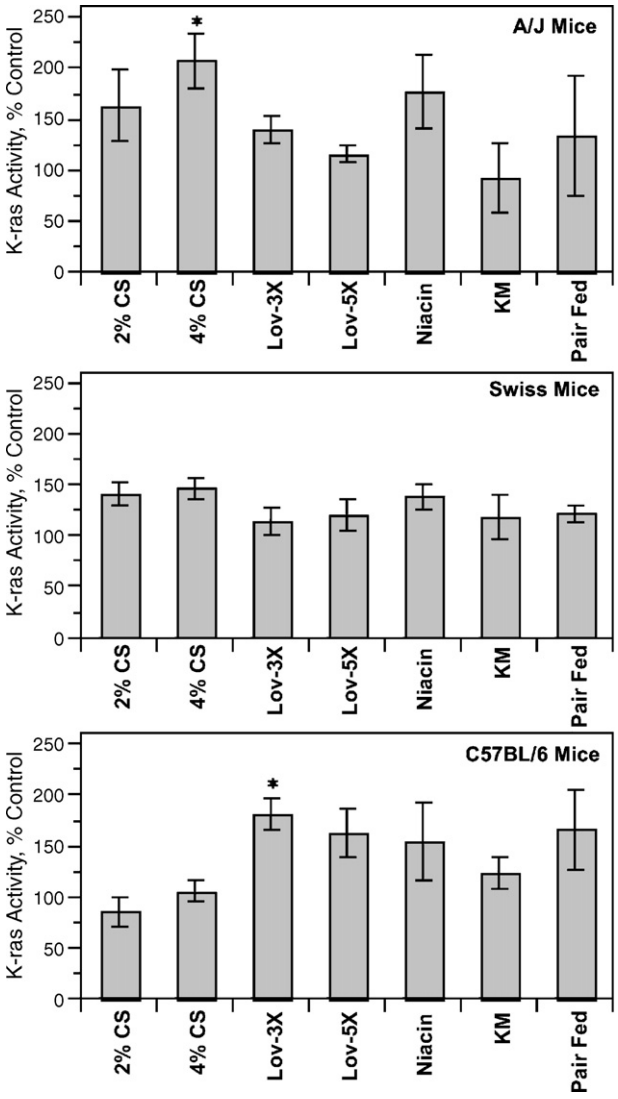

Fig. 4 – K-ras activity as a percent of control activity in mice treated with hypocholesterolemic agents for 3 weeks. Treatment groups include control (considered 100% activity), 2 or 4% cholestyramine (CS), lovastatin 25 mg/kg i.p. three or five times per week (Lov-3X or Lov-5X), 1% niacin, 5% konjac mannan (KM) or pair-fed. Bars are mean ± S.E.M. Each bar represents the mean from five animals, except in the Lov-5x and Pair-fed A/J mice, where $n = 4$. Seven different gels (one for each of these treatment groups) were run with the same strain-specific control samples on each gel for each of the mouse strains. A two-tailed t-test was used to evaluate statistically differences between treatment and control samples on each gel for each mouse strain. A/J mice: asterisk (*) denotes significantly higher than control ($p = 0.02$). In the 2 and 4% CS groups, a significant linear correlation was found between CS dose and activated K-ras protein level ($p < 0.001$, Pearson correlation). Swiss mice: no significant differences. C57BL/6 mice: asterisk (*) denotes significantly higher than control ($p = 0.009$).

p-values ranging from 0.4851 and 0.815 (data not shown). A similar analysis was done for Lov-3X treated mice, again showing no significant correlations between percent body weight change and activated K-ras level in any of the three mouse strains. Thus, weight loss is not a major factor in modulating K-ras activation in mouse lung.

4. Discussion

Although our study examined four hypocholesterolemic agents, only KM produced a significant lowering of serum cholesterol compared to control in two of the three mouse strains studied. KM is a viscous dietary fiber isolated from the tuber of the Japanese Konjac (Amorphophallus) plant. We used a purified food grade preparation called Propol A^{TM} (Shimizu Chemical Company, Hiroshima, Japan). It is a long chain polymer of glucose and mannose moieties in a 1:1.6 molar ratio joined by β-1–4 linkages with a molecular weight of approximately 2 million. KM has been found to reduce serum cholesterol significantly in rats made hypercholesterolemic [17,18] and in humans [13,19]. KM has been included in some dietary supplements with cholesterol-lowering claims. To the best of our knowledge, ours is the first report to demonstrate a significant cholesterol-lowering effect of KM in mice. The other published mouse study [20] examined the effect of KM on gall stones in mice, and showed a reduction in cholesterol of about 9%, which was not statistically significant. Our reductions of cholesterol with KM feeding of 28% (Swiss mice) or 41% (A/J mice) were much larger and statistically significant. It is of note that cholesterol lowering with KM occurred without any significant changes in activated K-ras levels. This would suggest that at least in lung one would expect no effect of KM on K-ras induced cancers (when K-ras is mutated) or on cancer prevention (wild-type K-ras).

K-ras activity is a key parameter both in tumor development (when mutated) and increasing evidence shows a role also in tumor suppression in lung [4–6]. It is interesting to note all of the hypocholesterolemic agents we studied tended to increase K-ras activity over control (with the exception of KM). Most of these changes were not statistically significant, but Lov, CS, and niacin increased K-ras activity over control in 14 of 15 cases, suggesting a possible trend toward increased activity. In retrospect, inclusion of three to five additional mice in each treatment group might have resulted in some of these results reaching statistical significance. This tendency toward increased K-ras activity may indicate a distinctive metabolic effect of Lov, CS, and niacin resulting in consistent increases in K-ras activity. Such increased activity might be tumor protective (as in lung) or could be tumor enhancing in other organs in which the role of wild-type K-ras and tumor development has not been studied.

CS significantly increased activated K-ras in A/J mice. Normal mouse lung tissue was used in our study, thus K-ras would be expected to act as a tumor suppressor in this context [4–6,21]. However, published studies in colon and mammary gland show CS to be a tumor promoter [22,23]. This may seem at odds with the conception of wild-type K-ras as a tumor suppressor in normal lung. However, there are several other factors to consider in interpreting this finding. First, animals in the cited studies were treated with chemical carcinogens to induce tumors. This would create a situation where ras is first mutated by chemical carcinogens. Perhaps in this special circumstance, further increases in K-ras activity induced by CS might be a liability. Second, a tumor suppressive effect for wild-type K-ras has thus far only been shown in lung, and may be unique to this tissue.

Lov-3X produced a significantly elevated level of activated K-ras, but only in C57BL/6 mice. This is consistent with several recent epidemiological studies showing decreased mortality from selected cancers in patients treated long-term with an HMG-CoA reductase inhibitor [7–9]. However, most reports have noted no effect of HMG-CoA reductase inhibitors on cancer [24–32]. A recent metanalysis and newly published large case–control study reported no benefit from statins in altering breast cancer risk [33] or colon cancer risk [34]. No published human epidemiological studies have specifically examined the effect of statins on lung cancer incidence. Considering that K-ras activity was affected by Lov in only C57BL/6 mice, any protective effect in humans, if present, would likely be highly influenced by genetic differences.

In summary, we noted a tendency of Lov, niacin, and CS each to increase K-ras activity in lung. We observed as well a number of mouse strain specific effects, illustrative of the importance of genetic background as a modifier of responses to the hypocholesterolemic agents. Only KM was effective in lowering cholesterol, with the effect confined to Swiss and C57BL/6 mice. Ours is the first report to show effective serum cholesterol reduction by KM in mice. Interestingly, KM was also the only hypocholesterolemic agent to have no significant effect on K-ras activity. CS produced elevated K-ras activity in A/J mice. Lov-3X increased levels of activated K-ras in C57BL/6 mice, consistent with a minority of human studies showing a protective effect of HMG-CoA inhibitors against cancer development. By inclusion of a pair-fed control group, we can state with confidence that the Lov effects in this study were not due to nonspecific effects of weight loss. Further exploration of the mechanism by which K-ras may act as a suppressor or differentiating agent in lung is warranted.

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