

Regulation of Mevalonate Synthesis in Rat Mammary Glands by Dietary n-3 and n-6 Polyunsaturated Fatty Acids¹

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Abstract

It is well established that dietary n-6 polyunsaturated fatty acids (PUFAs) enhance rat mammary tumor development whereas n-3 PUFAs inhibit it, yet the mechanisms are unclear. The objective of this study was to investigate a mechanism by which n-3 and n-6 PUFAs could modulate mammary carcinogenesis. Female Sprague Dawley rats were fed diets containing either menhaden (n-3) or safflower oil (n-6) in a 7% fat diet for 1 week. In comparison to the n-6 diet, the n-3 diet significantly reduced the activity and levels of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase in mammary glands, thereby suppressing the formation of mevalonate. In addition to being essential for cholesterol biosynthesis, mevalonate is also required for DNA synthesis and may be involved in malignant transformation. Serum cholesterol was lower in the n-3 group than in the n-6 group (1.91 ± 0.18 versus 2.61 ± 0.37 mM; $P < 0.01$). Extrahepatic tissues meet most of their cholesterol requirements from circulating cholesterol, and the internalized cholesterol down-regulates HMG-CoA reductase. Thus, the concomitant decrease in serum cholesterol and mammary gland HMG-CoA reductase levels suggests that changes in circulating cholesterol levels do not solely determine the activity of extrahepatic reductase. We conclude that the mevalonate pathway may be a mechanism through which different types of dietary fat modulate breast cancer development.

Introduction

Several studies have suggested that dietary fish oils may confer protection against human breast cancer (1–3). Animal studies have shown consistently that diets rich in n-3 PUFAs³ from fish oils (e.g., eicosapentaenoic acid and docosahexaenoic acid) inhibit mammary tumor development, whereas diets rich in n-6 PUFAs from vegetable oils (e.g., linoleic acid) enhance mammary tumor development (4–6). Eicosapentaenoic acid and docosahexaenoic acid also inhibit the growth of human breast cancer cells *in vitro* (7). Several potential mechanisms for the effects of dietary fat on mammary tumorigenesis have been proposed and reviewed extensively, yet the molecular mechanism still remains unclear (8, 9). One mechanism that has been proposed for the dietary fat-breast cancer association in humans relates to changes in serum cholesterol (10). Although animal studies relating serum cholesterol to mammary tumor development have not been consistent (11–13), rats fed fish oils do show significant reductions in circulating cholesterol levels (14). HMG-CoA reductase (EC

1.1.1.34) is the rate-limiting enzyme in the cholesterol biosynthetic pathway and catalyzes the conversion of HMG-CoA to mevalonate. Mevalonate is also an essential intermediate for the synthesis of isoprenoids involved in the membrane localization and subsequent activation of growth-regulatory proteins such as p21ras (15). Furthermore, mevalonate is required by mammalian cells for entry into the S phase of the cell cycle (16–18). Dietary isoprenoids (19) and drugs (20–23) that inhibit mevalonate synthesis are known to inhibit tumorigenesis both *in vitro* and *in vivo*. The objective of the present study was to determine whether the known effects of dietary n-6 and n-3 PUFAs on rat mammary tumorigenesis could occur via changes in mevalonate synthesis in the mammary gland. Although n-3 PUFAs have been shown to decrease HMG-CoA reductase activity in the liver (24), their effects on extrahepatic reductase are not known.

Materials and Methods

Materials. All reagents for electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA). The rabbit polyclonal anti-HMG-CoA reductase antibody was a gift from Dr. D. G. Hardie (University of Dundee, Scotland; Ref. 25), and the phosphoprotein phosphatase was a gift from Dr. J. H. Shand (Hannah Research Institute, Scotland, Ref. 26). 3-Hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA and [5-³H]mevalonolactone were from DuPont-New England Nuclear (Mississauga, Ontario, Canada), and all other reagents and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals and Diets. Female Sprague Dawley rats were purchased from Charles River Laboratories (St. Constant, Quebec, Canada) at 43 days of age and housed at 23°C and 50% humidity with a 12-h light/dark cycle. Animals were maintained on a standard AIN-93G control diet (Ref. 27; Dyets, Bethlehem, PA) for 1 week. They were then randomized into two groups ($n = 7$) and fed for 1 week diets in which the 7% soybean oil in the AIN-93G diet was replaced by 1% soybean oil (to ensure adequate amounts of linoleic acid) plus either 6% safflower oil or 6% menhaden oil (27). At the end of the experiment, rats were anesthetized, blood samples were taken by cardiac puncture, and animals were sacrificed by cervical dislocation. Serum was prepared from blood samples and stored at –70°C prior to analysis for total cholesterol using a kit from Boehringer Mannheim (Laval, Quebec, Canada).

Preparation of Microsomes. Liver and mammary gland microsomes were prepared by the method of Ness *et al.* (28). This procedure allows for the isolation of the full-length M_r 97,000 HMG-CoA reductase. Protein was determined using the Bio-Rad protein dye-binding assay according to the manufacturer's protocol, with BSA as a standard.

Immunoblotting. Microsomal proteins were solubilized in Laemmli buffer containing 8 M urea, heated for 5 min at 95°C, electrophoresed in 7.5% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. After incubating with anti-HMG-CoA reductase (1:2000), membranes were probed with horseradish peroxidase-conjugated anti-rabbit antibody (1:5000), and signals were detected by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). The M_r 97,000 HMG-CoA reductase band was identified by comparison with the migration of prestained molecular weight markers. Total proteins were stained with Coomassie blue to ensure equal loading.

Enzyme Activity. The activity of HMG-CoA reductase was determined using the radiochemical assay described by Ness *et al.* (29), and expressed as picomoles of mevalonolactone formed per minute and per milligram of mi-

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³The abbreviations used are: PUFA, polyunsaturated fatty acid; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA.

crossosomal protein. Phosphoprotein phosphatase was added to the assay buffer as described previously (26) to ensure measurement of total enzyme activity.

Results

After 1 week of feeding AIN-93G diets containing either menhaden or safflower oil, food intake and body weights did not differ between the two groups (data not shown). Serum cholesterol levels were significantly lower in the n-3 group (1.91 ± 0.18 mM) than in the n-6 group (2.61 ± 0.37 mM); $P < 0.01$ (Fig. 1). Levels of immunodetectable HMG-CoA reductase protein were lower in the mammary glands and livers of animals fed the menhaden oil diet compared to those fed the safflower oil diet, as shown in the representative Western blots in Fig. 2. The activity of HMG-CoA reductase in the mammary glands and livers is shown in Fig. 3. In rats fed the n-3 PUFA diet, the specific activity was significantly lower than those fed the n-6 PUFA diet in both the mammary glands (64.6 ± 4.1 versus 101.7 ± 7.1 pmol/min/mg; $P < 0.005$) and livers (176.1 ± 26.2 versus 312.0 ± 53.3 pmol/min/mg; $P < 0.05$).

Discussion

In the present study, we investigated a mechanism by which dietary fats rich in either n-3 or n-6 PUFAs could modulate mammary

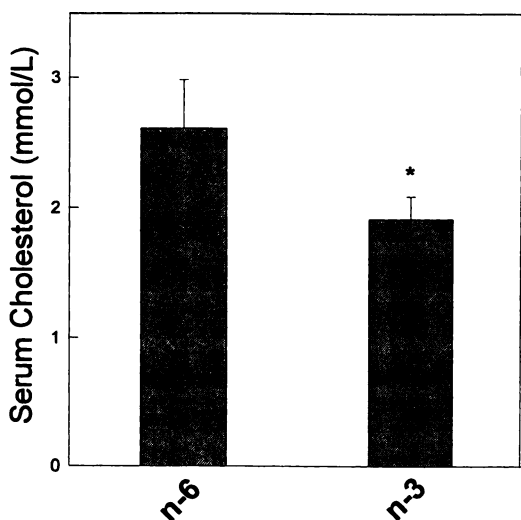
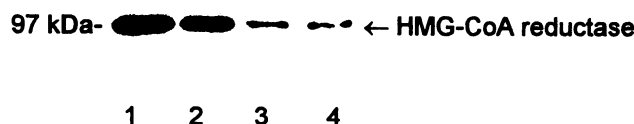


Fig. 1. Total serum cholesterol from rats fed n-6 or n-3 PUFA diets. *, significantly different; $P < 0.01$. Data are means; bars, SE.

A.



B.

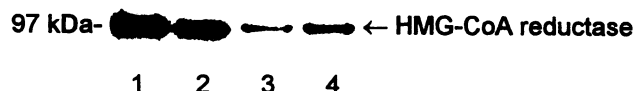


Fig. 2. Immunoblot of HMG-CoA reductase; representative samples of mammary gland microsomes (A) and liver microsomes (B) from rats fed n-6 (Lanes 1 and 2) or n-3 (Lanes 3 and 4) PUFA diets.

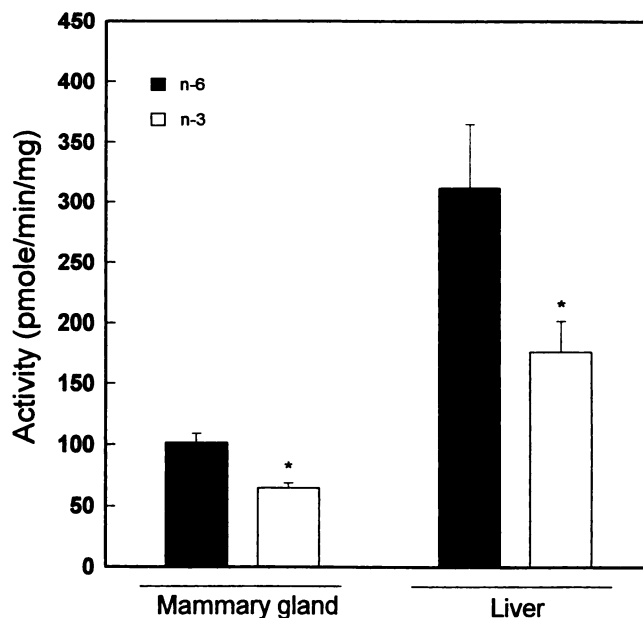


Fig. 3. Total activity of HMG-CoA reductase from mammary gland and liver microsomes of rats fed either n-6 or n-3 PUFA diets. *, significantly different from the n-6 group; $P < 0.05$. Data are means; bars, SE.

carcinogenesis. Our results demonstrate that HMG-CoA reductase activity and levels are significantly lower in mammary glands of rats fed an n-3 PUFA diet than in those fed an n-6 PUFA diet. Inhibitors of HMG-CoA reductase are known to inhibit tumorigenesis both *in vitro* and *in vivo* (21–23), as well as the proliferation of normal and neoplastic mammary epithelial cells (21). The findings of the present study, therefore, suggest that regulation of mevalonate synthesis in preneoplastic mammary epithelial cells by dietary fats may explain, at least in part, their modulating effects on mammary carcinogenesis and mammary tumor growth.

Rao *et al.* (30) have suggested that stimulation of *de novo* cholesterologenesis in the mammary gland may explain the promoting effects of a high-fat corn oil diet, and studies in other tissues and cells have also suggested an important role for cholesterol biosynthesis in tumor development (31). However, mevalonate not cholesterol *per se*, is required by mammalian cells for entry into the S phase of the cell cycle (16–18), a notion that supports our current findings. Nevertheless, cholesterol biosynthesis may be an approximate measure of mevalonate synthesis. Mevalonate is also a precursor of farnesyl PP_i that is required for the processing, membrane localization, and subsequent activation of p21ras (32). Inhibition of Ras processing may inhibit malignant transformation (33). Singh *et al.* (34) have shown recently that dietary menhaden oil inhibits colon tumorigenesis and p21ras membrane localization. Our observations support their hypothesis that this inhibition occurs at the level of HMG-CoA reductase.

As reported by others (14), in the present study menhaden oil led to lower levels of serum cholesterol than safflower oil. We attribute this difference to changes in hepatic HMG-CoA reductase, because the liver is the major site of lipoprotein synthesis and largely determines the levels of circulating cholesterol (35). Previously, we have shown that raising serum cholesterol by dietary cholesterol inhibits mammary carcinogenesis (11). Others have shown that lowering serum cholesterol by cholestyramine increases the incidence of mammary tumors (12). The authors of these two reports speculated that the increase or decrease in serum cholesterol leads to a decrease or increase, respectively, in mammary gland cholesterologenesis, because extrahepatic tissues meet most of their cholesterol requirements by internalizing circulating lipoproteins, the carriers of cholesterol in the

blood (36). The internalized cholesterol suppresses *de novo* cholesterologenesis by down-regulating HMG-CoA reductase. In the present study, however, the decrease in serum cholesterol by menhaden oil was associated with a concomitant decrease in HMG-CoA reductase in the mammary glands, suggesting that levels of circulating cholesterol are not solely responsible for the activity of the extrahepatic enzyme. Thus, changes in serum cholesterol alone are unlikely to predict tumor development. Rather, the effects of such changes on extrahepatic mevalonate synthesis may correlate better with tumorigenesis. Indeed, dietary cholesterol raises serum cholesterol, whereas dietary menhaden oil lowers serum cholesterol, yet both of these dietary factors inhibit HMG-CoA reductase and both inhibit mammary tumorigenesis (6, 11). The mechanism by which n-3 and n-6 PUFAs regulate mammary gland HMG-CoA reductase is currently being investigated.

In summary, we report that mevalonate synthesis is inhibited in the mammary glands of rats fed an n-3 PUFA diet compared to an n-6 PUFA diet. Our findings suggest that changes in mevalonate synthesis may account for the inhibitory effects of n-3 PUFAs and/or the promoting effects of n-6 PUFAs on mammary carcinogenesis.

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