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Elevation of serum cholesterol levels in mice by the antioxidant butylated hydroxyanisole

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Abstract—The food antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are structurally related to the hypocholesterolemic drug probucol. The purpose of this study was to determine if BHA can lower serum cholesterol levels as is observed with probucol. Treatment of mice with 0.75% BHA in their feed for 10 days resulted in a significant ($P \leq 0.01$) elevation of serum cholesterol levels. This effect contrasts with the cholesterol-lowering effect of probucol. Further, the degree of cholesterol elevation was comparable to that observed in mice administered 3% cholesterol in their feed for 7 days. The enzyme acyl CoA:cholesterol acyltransferase (ACAT) was decreased significantly ($P \leq 0.01$) in liver microsomes from BHA-treated mice. In contrast, hepatic microsomal ACAT activity was increased significantly ($P \leq 0.01$) in cholesterol-fed mice. These results suggested that the increased serum cholesterol observed in BHA-treated mice was not accompanied by an increase in hepatic cholesterol levels. Indeed, hepatic microsomal cholesterol levels were reduced in BHA-treated mice, but were increased significantly ($P \leq 0.01$) in cholesterol-fed mice. These results demonstrate that the common food additive BHA elevates serum cholesterol levels by a mechanism that apparently involves the decreased uptake of cholesterol by the liver.

Elevated serum cholesterol levels have been identified as a major risk factor in the development of atherosclerosis [1]. Several lines of evidence suggest that oxidized low density lipoprotein (LDL)* cholesterol is the primary contributor of cholesterol to macrophages resulting in their conversion to atherogenic foam cells [2–4]. Accordingly, significant effort has been expended toward the development of drugs that either lower serum cholesterol levels or protect against the oxidation of LDL cholesterol.

Probucol is an anti-atherogenic drug that both lowers serum cholesterol levels and protects against LDL cholesterol oxidation [5–7]. Probucol is thought to decrease serum cholesterol levels by increasing the uptake of high density lipoprotein (HDL) cholesterol by the liver [6]. Probucol is structurally related to the antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) that are extensively used as food additives [8, 9] and, accordingly, are consumed in appreciable quantities [8]. The purpose of the present study was to determine whether BHA altered cholesterol homeostasis as occurs with probucol.

Materials and Methods

Animal treatment. Adult male CD-1 mice were purchased from Charles River Laboratories and were 8- to 9-weeks-old and weighed 36 ± 2 g at the initiation of treatment. Control mice were provided 5 g of rodent feed per mouse daily (Agway Prolab). Each treated mouse received daily 5 g of feed containing either 0.75% BHA (Sigma) or 3% cholesterol (Sigma). The BHA and corresponding control treatments were terminated after 10 days, while cholesterol and corresponding control treatments were terminated after 7 days. Mice were not fasted prior to termination.

Mice were killed by cervical dislocation. Blood was immediately sampled by cardiac puncture and serum was prepared by allowing the blood to clot overnight at 4° followed by centrifugation. Livers were excised, minced in ice-cold 1.15% KCl and homogenized in buffer [0.01 M HEPES (pH 7.4), 1 mM EDTA, 10% glycerol]. Microsomes were prepared by differential centrifugation [10] and the microsomal pellet was resuspended in buffer [0.1 M potassium phosphate (pH 7.4), 0.1 mM EDTA, 20% glycerol]. Protein concentration of the preparations was determined according to Bradford [11] using commercially available reagent (Biorad) and bovine serum albumin (Sigma) as a standard.

Cholesterol measurements. Serum total cholesterol was determined by the oxidase method [12] using commercially available reagents (Sigma). Hepatic microsomal cholesterol was determined using a modification of this procedure as described elsewhere [13]. Briefly, hepatic microsomes were mixed with the oxidase reagents at 4° and absorbance of the mixture was measured at 500 nm. The mixture was then incubated for 10 min at 37° and the absorbance at 500 nm measured again. The difference in absorbance was indicative of the cholesterol content of the microsomes.

Acyl CoA: cholesterol acyltransferase (ACAT) activity assays. ACAT activity was determined as described elsewhere [14] with the following modifications. ACAT activity was measured in a 400 μL incubation mixture

Table 1. Effects of BHA and cholesterol treatment on male CD-1 mice

Treatment	Serum cholesterol* (mg/dL)	Mouse weight (g)	
		Initial	Terminal
Control	123 ± 17	36 ± 1	36 ± 1
BHA	154 ± 18†	36 ± 1	35 ± 1
Control	123 ± 24	37 ± 2	37 ± 2
Cholesterol	161 ± 31†	35 ± 2	34 ± 2

Mice, 8- to 9-weeks-old at the initiation of treatment, were administered BHA or cholesterol in their diet at a concentration of 0.75 or 3% of their feed, respectively. After 10 days (BHA) or 7 days (cholesterol) mice were killed and tissues prepared. All data are presented as means ± SD, N = 10.

* Serum cholesterol levels were measured by the cholesterol oxidase method [11].

† Significantly different from the respective control ($P \leq 0.01$), Student's *t*-test.

* Abbreviations: LDL, low density lipoprotein; BHA, butylated hydroxyanisole; and BHT, butylated hydroxytoluene.

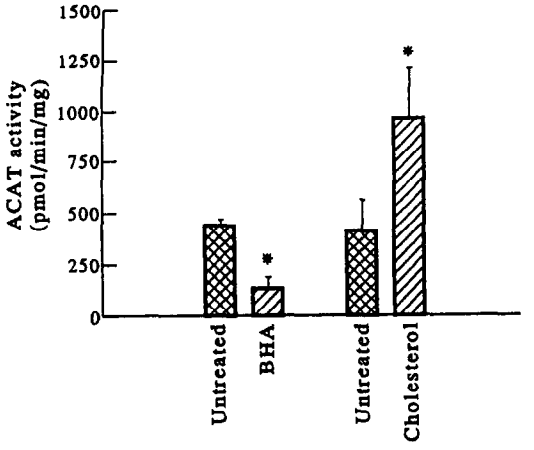

Fig. 1. Hepatic ACAT activity following treatment of mice with either BHA or cholesterol. Following treatment of mice with either BHA (0.75% in feed for 10 days) or cholesterol (3% in feed for 7 days), ACAT activity was measured in liver microsomes as described in Ref. 14 with some modifications (see Materials and Methods). Values are means ± SD (N = 5). Key: (*) significantly different from the control at $P \leq 0.01$ (Student's *t*-test).

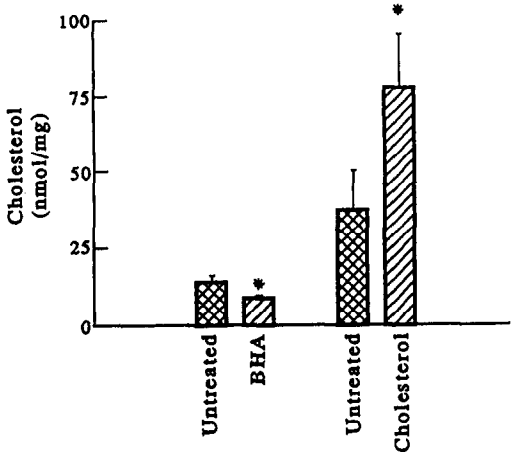

Fig. 2. Hepatic microsomal cholesterol levels following treatment of mice with either BHA or cholesterol. Following treatment of mice with either BHA (0.75% in feed for 10 days) or cholesterol (3% in feed for 7 days), hepatic microsomal cholesterol levels were measured by the cholesterol oxidase method [12] as modified [13]. Values are means ± SD (N = 5). Key: (*) Significantly different from the control at $P \leq 0.01$ (Student's *t*-test).

containing 30 μg microsomal protein, 20 nmol fatty acid-free bovine serum albumin, 0.1 M potassium phosphate buffer (pH 7.4), 1 mM EDTA and 2 mM diothiothreitol (DTT). The mixture was preincubated for 6 min at 37° before initiating the reaction by adding 18 nmol [^{14}C]-palmitoyl-CoA (5.5 mCi/mmol). The reaction was terminated after 6 min by adding 5 mL chloroform:methanol (2:1, v/v). Lipids were extracted into the organic solvent, separated by thin-layer chromatography, and quantitated by scintillation spectrometry [14].

Results and Discussion

Administration of BHA to mice in their diet for 10 days resulted in a 25% elevation in serum cholesterol levels (Table 1). This elevation in serum cholesterol was comparable to that obtained when mice were administered 3% cholesterol in their diet for 7 days (Table 1). The administration of BHA had no adverse effect on the mice as judged by food consumption rates and weight change (Table 1).

The increase in serum cholesterol levels following BHA administration could be due to effects of the compound on the rates of hepatic cholesterol synthesis or elimination. Increased cholesterol synthesis or decreased elimination would both be accompanied by an increase in the hepatic enzyme ACAT which is responsible for the esterification of hepatic free cholesterol [15]. Hepatic ACAT activity was actually decreased in BHA-treated mice (Fig. 1). In contrast, cholesterol-fed mice had significantly elevated ACAT activity (Fig. 1).

The reduced ACAT activity in BHA-fed mice could be due to either a reduction in the amount of ACAT enzyme present in the hepatic microsomes or a reduction in microsomal cholesterol which is the source of cholesterol in the ACAT assay. Analysis of hepatic microsomes revealed that cholesterol content was reduced significantly following BHA treatment (Fig. 2). Again, this effect stands in contrast to the effect of cholesterol feeding which significantly elevated hepatic microsomal cholesterol (Fig. 2).

These results show striking contrasts between the effects of BHA and the known effects of probucol on cholesterol homeostasis. While probucol reduces serum cholesterol levels due to increased hepatic uptake [6], BHA elevates serum cholesterol apparently due to decreased hepatic uptake. The increase in serum cholesterol following BHA treatment was presumably not due to increased synthesis or decreased elimination since these effects would have resulted in increased hepatic ACAT activity and increased hepatic cholesterol levels as was observed with cholesterol feeding. Elevated serum cholesterol levels have also been observed following treatment with the related antioxidant BHT [16]. However, despite this increase, BHT protected somewhat against artherosclerosis, apparently due to its antioxidant effects. These results demonstrate that the common food additives BHA and BHT affect cholesterol homeostasis differently than does the related drug probucol.

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