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Cholesterol-rich diets have different effects on lipid peroxidation, cholesterol oxides, and antioxidant enzymes in rats and rabbits

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The objective of this study was to compare the effect of cholesterol feeding of rats and rabbits. The levels of lipid peroxidation products and oxysterols in the plasma of the two species plus the antioxidant enzyme activities in the liver and erythrocytes were measured to explain their different susceptibilities to atherosclerosis. Our study showed that rats are less susceptible than are rabbits to the atherogenic effect of a cholesterol-rich diet because of differences in lipid peroxidation products as well as antioxidant enzymes activities in their livers. In rabbits, cholesterol feeding produced severe hypercholesterolemia (43-fold increase) and increased plasma and liver lipid peroxidation. Total as well as the individual oxysterol contents of 7α-, 7β-hydroxycholesterol, α-epoxy, β-epoxycholesterol, cholestanetriol, 7-keto, and 27-hydroxycholesterol significantly increased in the plasma of hypercholesterolemic (HC) rabbits. Erythrocyte glutathione peroxidase (GSH-Px) activity significantly decreased whereas catalase activity significantly increased in HC rabbits. In rats cholesterol feeding increased the plasma cholesterol only twofold and had no effect on plasma or liver lipid peroxidation. Only 7α- and 7β-hydroxycholesterol increased and no change was observed in any of the antioxidant enzymes activity in the erythrocytes. Although cholesterol feeding caused a 10-fold increase of liver cholesterol as ester in both rats and rabbits, the antioxidant enzyme GSH-Px and catalase activities in the liver significantly increased in rats but significantly decreased in rabbits. The increase of GSH-Px and catalase activities in the liver of cholesterol fed rats could have a protective role against oxidation, thus preventing the formation of lipid peroxidation and oxysterols. (J. Nutr. Biochem. 11:293–302, 2000) © *Elsevier Science Inc. 2000. All rights reserved.*

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Introduction

Feeding a cholesterol-supplemented diet to rabbits produced hypercholesterolemia and vascular atherosclerotic lesions, caused increased lipid peroxidation (LPX), exposed the animals to oxidative stress,[1,2] and significantly increased the level of plasma oxysterols.[3]

Rats, however, are relatively resistant or less sensitive to the atherogenic effect of a cholesterol-supplemented diet. When rats were fed a diet that contained 1% cholesterol for 8 weeks, their plasma peroxidation products did not increase compared with rats fed a cholesterol-free diet.[4] Cholesterol feeding in rats also decreased the serum thromboxane (TXA_2) level whereas in rabbits it produced an opposite effect and increased the TXA_2 synthesis by platelets.[5]

Oxysterols represent one of the primary pro-atherogenic components of a cholesterol-rich diet.[6–8] Cholesterol per se has little atherogenic or cytotoxic effect, as shown by venous infusion[9,10] and in vitro studies, when compared with oxysterols.[11,12] The barrier function of endothelial cells in culture was not significantly affected when exposed to cholesterol-enriched low density lipoproteins (LDL), whereas triol (cholestane-3β, 5α, 6β-triol)-enriched LDL resulted in a significant loss of barrier function,[13] indicating that oxysterols rather than cholesterol promoted atherogen-

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esis. Rabbits fed a concentrate of oxysterols by gastric gavage showed arterial wall damage within 24 hr, while atheromatous lesions developed with prolonged feeding.[14]

Oxidative stress is one of the causative factors that link hypercholesterolemia with the pathogenesis of atherosclerosis. This stress results from the imbalance between the production of free radicals and the effectiveness of the antioxidant defense system.[15] The activity of free radicals is countered by a system of antioxidant defenses, of which vitamin E is the major chain breaking lipophilic antioxidant in tissues and plasma. In addition to vitamin E (α-tocopherol), tissues and erythrocyte enzymes contribute to the cell antioxidant defense mechanism. These include superoxide dismutase (SOD), which catalyzes dismutation of superoxide anions into hydrogen peroxide (H_2O_2), and catalase and glutathione peroxidase (GSH-Px), which detoxify H_2O_2 and convert lipid hydroperoxides to nontoxic alcohol. Disorders in these erythrocyte enzyme activities have been reported in subjects with cardiovascular disease.[16]

The addition of free radical scavengers to a cholesterol-supplemented diet[3,17–19] significantly decreased the plasma LPX products as well as the severity of atherosclerotic lesions in rabbits. The addition of antioxidants to the cholesterol diet of hypercholesterolemic (HC) rabbits also decreased the oxysterol concentration in the plasma as well as the severity of atherosclerosis although hypercholesterolemia still existed.[3] This indicated that LPX and oxysterols were among the promoting factors for atherosclerosis.

The objective of this study was to examine the influence of a cholesterol-supplemented diet on the activities of the antioxidant enzymes SOD, catalase, and GSH-Px in livers and erythrocytes as well as its influence on the levels of plasma oxysterols and LPX in rats and rabbits. These parameters may clarify some of the factors that make rats resistant to the atherogenic effect of a cholesterol-supplemented diet.

Materials and methods

Animals and diets

Twelve New Zealand white male rabbits ranging in weight from 2,157 to 2,304 g and 12 Sprague-Dawley male rats ranging in weight from 186 to 221 g were housed individually in stainless steel mesh-bottomed cages. The rabbits and rats were divided into two groups of six animals each. One group of six rabbits was fed a nonpurified standard rabbit diet (Purina Mills, Inc., St. Louis, MO USA; basal diet) and the six rabbits in the experimental group were fed the basal diet plus 1% pure cholesterol. The control group of rats was fed a pelleted basal rat diet (Teklad, Madison, WI USA). The experimental group received the same basal diet plus 1% pure cholesterol. Pure cholesterol was prepared by six crystallizations of USP cholesterol from 95% ethanol and was kept under nitrogen (N_2) at −20°C. Its purity was regularly checked by gas chromatography, which indicated that it did not contain a detectable amount of oxysterols.

The cholesterol diets were prepared by dissolving the cholesterol in peroxide-free ether, spraying it as a fine mist over the diet, and allowing the ether to evaporate in a closed hood under N_2 at room temperature. The cholesterol-free (basal) diet was sprayed with an equal volume of peroxide-free ether. The diets were prepared fresh every 3 days. Any remaining rations in the feeding bins were discarded daily. After 1 and 2 months on the respective diets all the animals were fasted overnight. Rabbits were anesthetized by intramuscular injection of ketamine (44 mg/kg) and xylazine (5 mg/kg), and 5 mL blood was withdrawn from the ear vein. Rats were anesthetized by ether and 3 mL blood was withdrawn by heart puncture. All blood samples were collected in plastic tubes containing ethylenediamine tetraacetic acid (EDTA; 1 mg/mL blood) as anticoagulant and butylated hydroxy toluene (BHT; 50 μg/mL blood) as antioxidant. After 4 months the feeding experiment was terminated and all animals were fasted overnight. Rabbits and rats were anesthetized and blood was collected by heart puncture. Rabbits were euthanized by pentobarbital injection (100 mg/kg) and rats were exposed to carbon dioxide gas. The livers were excised immediately and washed with ice-cold 0.25 M sucrose solution. A portion of each liver was prepared for antioxidant enzymes assay and LPX [as thiobarbituric acid reactive substances (TBARS)] measurement. The remaining liver was stored at −80°C for lipid analysis.

All animal protocols were approved by the University of Illinois at Urbana-Champaign Animal Care and Use Committee.

Chemicals and reagents

Nitroblue tetrazolium, SOD (3,100 units/mg protein), xanthine oxidase (0.65 unit/mg protein), diethylene triamine pentaacetic acid, reduced glutathione, reduced nicotinamide adenine dinucleotide phosphate (NADPH), and α-tocopherol (99.5% pure) were purchased from Sigma Chemical Co. (St. Louis, MO USA). Xanthine and disodium salt of bathocuproinedisulfonic acid were obtained from Aldrich Chemical Co. (Milwaukee, WI USA). Glutathione reductase (203.4 U/mg protein) was purchased from Fluka Chemie AG (Neu-Ulm, Switzerland). Cholesterol oxide standards, cholestane-3β, 5α, 6β-triol (cholestanetriol), 5-cholestene-3β, 25-diol (25-hydroxycholesterol), 5-cholestene-3β, 7β-diol (7β-hydroxycholesterol), 5β, 6β-epoxycholestan-3β-ol (β-epoxycholesterol), 5α, 6α-epoxycholestan-3β-ol (α-epoxycholesterol), 5-cholestene-3β, 20α-diol (20α-hydroxycholesterol), and 5-cholestene-3β-0l-7 one (7-ketocholesterol) were purchased from Sigma Chemical Co. 5-Cholestene-3β, 7α-diol (7α-hydroxycholesterol), and 5-cholestene-3β, 27 diol (27-hydroxycholesterol) were obtained from Steraloid (Wilton, NH USA). All other chemicals and reagents were of analytical or high performance liquid chromatography (HPLC) grade.

Lipid analysis

Total plasma cholesterol,[20] high density lipoprotein (HDL) cholesterol,[21] and triacylglycerol[22] were measured according to the instruction manuals accompanying the diagnostic kits obtained from Sigma Chemical Co. The lipid extracted from 1 mL plasma[23] was used for measuring the total lipid phosphorus.[24]

For liver lipid analysis, a 1 g portion of each liver was extracted[23] and the lipid residue was taken up in 10 mL chloroform in a 10 mL volumetric flask. Known aliquots of this lipid extract were used for measuring the free and total cholesterols. [25] Total lipid phosphorus[24] and triacylglycerol[26] were measured using Sigma kits.

Measurement of TBARS

LPX was measured flourometrically as plasma malondialdehyde by the thiobarbituric acid method.[27] The TBARS in liver tissue homogenate were also measured flourometrically according to the method of Yagi[28] as described by Prasad et al.[29] Tetramethoxypropane was used as a standard and the results were expressed as nmoles of malondialdehyde equivalents.

Measurement of selenium in plasma and erythrocytes

Selenium was analyzed at the Laboratory of Diagnostic Medicine of the College of Veterinary Medicine, University of Illinois at Urbana-Champaign. All the selenium in the sample was converted to the Se (IV) oxidation state by a two-step process. Samples were first ashed with $Mg(NO_3)_2/HNO_3$ solution and the resulting residue was dissolved in concentrated HCl. The resulting Se (IV) was combined with 4-nitro-0-phenylenediamine (NPD) to form 5-nitropiazselenol. The NPD complex was extracted into toluene and quantitated by gas chromatography using electrocapture detection procedure.[30]

Measurement of vitamin E (α-tocopherol) by HPLC method

All analyses of α-tocopherol were conducted by normal phase liquid chromatography. Briefly, the plasma samples were saponified in the presence of 0.2 mL 15% ascorbic acid, 2 mL ethanol containing 0.025% BHT, and 1 mL 40% KOH solution.[31] α-Tocopherol was extracted with hexane containing 0.025% BHT. The hexane phase was removed and dried over sodium sulfate anhydrous, and 20 μL was injected into the HPLC column. For tocopherol separation we used Phenomenex Prodigy silica 100 A° 5 μm, 4.6 × 150 mm column and an isocratic mobile phase of 2.5% tetrahydrofuran in isooctane pumped at 1.5 mL/min and 600 psi. The detection system was ultraviolet at 295 nm. The equipment used was an HP model 1050 pump, variable wavelength detector, and Rheodyne (Wilmington, DE USA) valve with 20 μL loop connected on HP Chemstation for quantitation.

Measurement of plasma oxysterols

Plasma oxysterols were determined using a quantitative gas chromatographic method. Briefly, to 500 μL plasma, 2 mL of 1 M KOH in methanol containing 0.05% BHT as antioxidant and 500 ng of 20-hydroxycholesterol (internal standard) were added. The tube was flushed with N_2 and samples were allowed to saponify at room temperature overnight.[8] To the saponified sample 4 mL of water were added and lipids were extracted twice with 3 mL hexane. The lipid extract was dried under N_2 and the residue was dissolved in 500 μL acetonitrile and vortexed, after which 500 μL deionized water was added and the solution vortexed again. The lipid residue in acetonitrile was passed through a C-18 cartridge (Waters Associates, Millford, MA USA) to separate oxysterols from other lipids, then further purified by HPLC, and finally analyzed by gas chromatography as previously described.[32]

Preparation of erythrocyte lysate for measurement of antioxidant enzymes

Blood collected in EDTA was centrifuged at 2,000 × g for 10 min at 4°C to separate plasma from the erythrocytes. Erythrocytes were washed three times with physiologic saline (0.9% NaCl) containing 0.5% of pyrogallol as antioxidant. The final hematocrit suspension was made up to 50% with the addition of distilled water containing 50 μg/100 mL BHT.[33] Aliquots of this lysate were used for measurement of the antioxidant enzymes.

The total hemoglobin content of the lysate was measured colorimetrically[34] using Drabkin's solution as color reagent and cyanometh hemoglobin as standard.

This lysate was used directly for the measurement of catalase and GSH-Px activity. For the assay of CuZn-SOD, 0.25 mL of the lysate was added to 1.75 mL water and 0.8 mL chloroform/ethanol mixture (3:5, v/v) and vortexed for 1 min.[35] The mixture was then centrifuged at 2,000 g for 20 min at 4°C and the supernatant was used for measurement of CuZu-SOD activity.

Preparation of liver tissues for measurement of the antioxidant enzymes

Livers were excised immediately, washed with ice-cold 0.25 M sucrose solution, and blotted dry. The livers were weighed, then chopped with scissors into ice-cold 0.25 M sucrose, which was then made to 4 volumes (w/v) with 0.25 M sucrose and homogenized for 10 sec using a Polytron homogenizer (Brinckman Instrument, Rexdale, Ontario, Canada) at a speed setting of 6.[36] The homogenate was centrifuged for 60 min at 100,000 g in a Beckman Model L3-50 ultracentrifuge at 4°C. The supernatant fraction was decanted and used to determine the GSH-Px and SOD activities. For catalase assay, the homogenate was centrifuged at 700 g for 10 min. Ethanol was added to the supernatant fraction to a final concentration of 0.17 M to decompose the inactive stable enzyme-substrate complex of catalase-H_2O_2. The mixture was placed on ice for 20 min, after which a 100 μL phosphate buffer (pH 7.0) containing 100 mL Triton X-100/L was added to 1 mL of the mixture.[36,37] Protein concentrations in the supernatants were measured[38] using bovine serum albumin as standard.

Measurement of SOD activity

SOD activity in the erythrocyte lysate, after being treated with $CHCl_3$/ethanol or the supernatant of liver homogenate, was measured by the method of Sun et al.[35] as described by Mantha et al.[39] One-half milliliter of different concentrations (30–300 ng/mL) of standard SOD was added to the standard tubes, and 0.5 mL of the diluted hemolysate (1:1,000) or the supernatant of the liver homogenate (10–250 μg protein) was added to the sample tubes. After incubation at 25°C for 20 min, the reaction was terminated by the addition of 1 mL of 0.8 mM cupric chloride. The formazan produced was measured spectrophotometrically at 560 nm. The percent inhibition and the SOD activity was calculated from the standard curve of the SOD enzyme as previously described.[40]

Measurement of catalase activity

Catalase activity was measured by the method of Aebi.[37] Two milliliters of the diluted hemolysate (1:1,000 by 50 mM phosphate buffer, pH 7.0) or the supernatant of the liver homogenate (200–500 μg protein) were added to a cuvette and the reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H_2O_2 in a phosphate buffer (50 mM, pH 7.0). The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm during the first 30 sec. The activity of catalase was expressed as *k* per second per gram of hemoglobin for erythrocyte lysate or *k* per second per milligram of protein for the liver homogenate, where *k* is the rate constant of a first-order reaction.

Measurement of GSH-Px activity

GSH-Px activity was measured by the method of Pagalia and Valentine[41] as described by Mantha et al.[39] For measuring the GSH-Px activity in the erythrocyte lysate, 100 μL of the lysate was diluted to 500 μL with phosphate buffer (50 mM, pH 7.0). Then 500 μL of double-strength Drabkin's reagent was added to convert all hemoglobin to stable cyanometh hemoglobin. Fifty microliters of that mixture or aliquots (50–200 μL) of the liver homogenate supernatant (200–500 μg protein) were added to the assay mixture and the reaction was started by the addition of 0.1 mL of 7.5 mM H_2O_2. Conversion of NADPH to $NADP^+$ was monitored continuously in a spectrophotometer at 340 nm for 3 min. GSH-Px

Table 1 Plasma lipids, vitamin E, selenium, and thiobarbituric acid reactive substances (TBARS) in rabbits fed basal or cholesterol-rich diets for 1, 2, and 4 months

	1 Month		2 Months		4 Months	
	Basal ($n = 6$)	Cholesterol ($n = 6$)	Basal ($n = 6$)	Cholesterol ($n = 6$)	Basal ($n = 6$)	Cholesterol ($n = 6$)
Total cholesterol (mmol/L)	0.84 ± 0.062	31.42 ± 0.23[d]	1.44 ± 0.19	46.11 ± 7.33[d]	1.17 ± 0.1	50.62 ± 11.0[d]
HDL cholesterol (mmol/L)	0.52 ± 0.11	0.36 ± 0.025	0.65 ± 0.018	0.51 ± 0.093	0.47 ± 0.12	0.25 ± 0.1[a]
Triglycerides (mmol/L)	0.42 ± 0.02	1.27 ± 0.17[c]	0.47 ± 0.005	1.81 ± 0.54[a]	0.69 ± 0.04	2.53 ± 0.8[c]
Phospholipids (mmol/L)	0.92 ± 0.14	5.86 ± 0.39[d]	0.76 ± 0.02	4.9 ± 1.07[b]	0.74 ± 0.11	6.36 ± 1.02[d]
TBARS (μmol MDA/L plasma)	1.6 ± 0.22	4.2 ± 0.84[a]	1.2 ± 0.40	3.17 ± 0.20[c]	0.80 ± 0.45	2.8 ± 0.70[b]
α-Tocopherol (μmol/mmol cholesterol)	ND	ND	ND	ND	7.65 ± 0.93	1.03 ± 0.19[d]
Selenium (μmol/L)	ND	ND	2.97 ± 0.09	3.06 ± 0.13	3.33 ± 0.12	3.43 ± 0.28
Body weight (kg)	3.1 ± 0.08	3.0 ± 0.12	3.75 ± 0.22	3.30 ± 0.25[a]	4.52 ± 0.32	3.25 ± 0.44[c]

Results are expressed as means ± SD of six animals per group. At each feeding period the mean values with a superscript letter a, b, c, or d are significantly different from their controls fed the basal diet at levels of $P < 0.05$, $P < 0.02$, $P < 0.01$, or $P < 0.001$, respectively.
HDL–high density lipoprotein. MDA–malondialdehyde. ND–not determined.

activity was expressed as nmoles of NADPH oxidized to $NADP^+$ per minute per gram of hemoglobin of the erythrocyte or minute per milligram of protein of the liver homogenate supernatant, using an extinction coefficient 6.22×10^6 for NADPH.

Statistical analysis

Results are expressed as means with their standard deviations. The statistical significance of differences between means was measured by Student's *t*-test using group analysis.

Results

Feeding a cholesterol-supplemented diet to rabbits increased the plasma total cholesterol more than 37-fold after 1 month and more than 43-fold after 4 months compared with rabbits fed the basal diet (*Table 1*). HDL cholesterol decreased in rabbits fed the cholesterol-supplemented diet, a decrease that was significant after 4 months, which indicated that the increase of plasma cholesterol was mainly within the LDLs [very low density lipoproteins (VLDL) and LDL]. Plasma phospholipid (PL) concentration increased more than 6-fold in rabbits fed the cholesterol diet for 1 and 2 months and that increased more than 8-fold after 4 months compared with rabbits fed the basal diet. Plasma triglyceride (TG) concentration in rabbits increased more than 3-fold after feeding cholesterol for 1 month, and that increase was 4- and 3.7-fold higher than rabbits fed the basal diet after 2 and 4 months, respectively. Feeding a cholesterol diet to rabbits for 1 month significantly increased the TBARS in the plasma, an increase that persisted at 2 and 4 months (*Table 1*). After 4 months on the respective diets, plasma α-tocopherol (vitamin E) concentration as related to the cholesterol concentration (μmole vitamin E/mmole cholesterol) significantly decreased from 7.65 ± 0.93 in rabbits fed the basal diet to 1.03 ± 0.19 in rabbits fed the cholesterol diet. Selenium concentration in the rabbit plasma (as μmole/L) was not significantly different between rabbits fed the cholesterol or basal diets (*Table 1*).

Table 2 shows that when rats were fed a cholesterol diet,

Table 2 Plasma lipids, vitamin E, selenium, and thiobarbituric acid reactive substances (TBARS) in rats fed basal or cholesterol-rich diets for 1, 2, and 4 months

	1 Month		2 Months		4 Months	
	Basal ($n = 6$)	Cholesterol ($n = 6$)	Basal ($n = 6$)	Cholesterol ($n = 6$)	Basal ($n = 6$)	Cholesterol ($n = 6$)
Total cholesterol (mmol/L)	1.93 ± 0.052	1.51 ± 0.09[a]	2.16 ± 0.18	2.62 ± 0.07[a]	2.99 ± 0.35	7.0 ± 1.27[c]
HDL cholesterol (mmol/L)	0.97 ± 0.015	0.84 ± 0.002[a]	1.16 ± 0.17	1.18 ± 0.16	2.09 ± 0.13	1.40 ± 0.33[b]
Triglycerides (mmol/L)	0.73 ± 0.08	0.70 ± 0.03	0.88 ± 0.05	0.93 ± 0.06	1.31 ± 0.12	1.48 ± 0.09
Phospholipids (mmol/L)	1.47 ± 0.1	1.21 ± 0.16	1.05 ± 0.2	1.23 ± 0.18	1.92 ± 0.25	2.13 ± 0.3
TBARS (μmol MDA/L plasma)	1.55 ± 0.12	1.50 ± 0.20	1.88 ± 0.38	1.75 ± 0.40	1.80 ± 0.36	1.82 ± 0.39
α-Tocopherol (μmol/mmol cholesterol)	ND	ND	ND	ND	6.52 ± 0.76	6.06 ± 1.37
Selenium (μmol/L)	ND	ND	6.73 ± 0.59	8.3 ± 0.19[a]	7.83 ± 0.1	8.9 ± 0.64[a]
Body weight (g)	370 ± 13.8	364 ± 16.5	434 ± 20.4	434 ± 20.7	502.5 ± 16.2	502 ± 22.6

Results are expressed as means ± SD of six animals per group. At each feeding period the mean values with a superscript letter a, b, or c are significantly different from their controls fed the basal diet at levels of $P < 0.05$, $P < 0.02$, or $P < 0.01$, respectively.
HDL–high density lipoprotein. MDA–malondialdehyde. ND–not determined.

Table 3 Lipid, selenium, and thiobarbituric acid reactive substances (TBARS) in liver tissues of rabbits and rats fed basal or cholesterol-rich diets for 4 months

	Rabbits		Rats	
	Basal	Cholesterol	Basal	Cholesterol
Total cholesterol (μmol/g liver)	8.55 ± 1.11	88.34 ± 16.0[b]	10.1 ± 0.9	106.5 ± 20.2[d]
Free cholesterol (μmol/g liver)	5.7 ± 0.67	18.9 ± 0.65[d]	5.2 ± 0.26	10.4 ± 3.6[a]
% Esterification	33.9 ± 2.2	78.1 ± 3.8[d]	47.4 ± 3.26	90.0 ± 3.4[d]
Triglycerides (μmol/g liver)	10.5 ± 2.48	4.63 ± 0.9[c]	4.2 ± 0.38	15.6 ± 4.63[c]
Phospholipids (μmol/g liver)	35.38 ± 2	33.25 ± 5.8	32.8 ± 2	32.5 ± 3.63
TBARS (nmol MDA/g liver)	14.2 ± 5.5	42.7 ± 11.5[c]	18.8 ± 5.9	22.9 ± 4.5
Selenium (nmol/g liver)	20.7 ± 5.2	20.8 ± 4.6	22.2 ± 2.1	20.7 ± 6.6

Results are expressed as means ± SD of six animals per group. Mean values with a superscript letter a, b, c, or d are significantly different from their controls fed the basal diet at levels of $P < 0.05$, $P < 0.2$, $P < 0.01$, or $P < 0.001$, respectively.
MDA–malondialdehyde.

their plasma total cholesterol did not significantly increase until after 2 months and the increase was only 1.2-fold higher than that of the control group, but that increase was 2.3-fold after 4 months of cholesterol feeding. HDL cholesterol decreased in the group of rats fed the cholesterol diet and that decrease was significant after 4 months. No significant differences were observed for the plasma TG, PL, TBARS, or vitamin E (as μmole/mmole cholesterol) between rats fed the cholesterol and basal diets at any time (*Table 2*). After 2 and 4 months of the feeding experiment the plasma selenium concentration was significantly higher in rats fed the cholesterol diet than in those fed the basal diet. The basal plasma concentrations of cholesterol, TG, and PL were higher in the control rats (*Table 2*) than in the control rabbits (*Table 1*).

Feeding cholesterol for 4 months increased the liver cholesterol 10-fold in both rabbits and rats. Cholesterol ester also represented 78% and 90% of the liver cholesterol in rabbits and rats, respectively, indicating an enhancement of cholesterol esterification by feeding cholesterol. The liver TG significantly decreased in rabbits whereas it significantly increased in rats fed cholesterol, but liver PL did not significantly change in either rats or rabbits fed cholesterol compared with their controls. Although TBARS significantly increased in the liver of rabbits fed cholesterol, no significant difference was observed for TBARS in the livers of rats fed the cholesterol diet. The liver selenium concentration was not significantly different between rats or rabbits fed cholesterol compared with their controls (*Table 3*).

Table 4 shows that the selenium concentration significantly decreased in the erythrocytes of the rabbits fed the cholesterol diet for 2 months (22.56 ± 0.41 nmole/g Hb in the controls vs. 15.3 ± 0.38 nmole/g Hb in the cholesterol group) and for 4 months (21.53 ± 2.4 nmole/g Hb in the controls vs. 13.6 ± 2.3 nmole/g Hb in the cholesterol group). On the contrary, there was no significant difference in the selenium concentration of the erythrocytes of rats fed either cholesterol or basal diets at any time (*Table 4*). The erythrocyte cholesterol:PL ratio (mole/mole) significantly increased only in rabbits fed the cholesterol diet for 4 months (1.86 ± 0.22) compared with the control group (1.1 ± 0.1).

The total plasma oxysterol concentration significantly increased in rabbits fed the cholesterol diet (6.8 ± 0.68 μmole/L) compared with their controls (1.53 ± 0.23 μmole/L; *Table 5*). The individual oxysterols, namely 7α, 7β, β-epoxy, α-epoxy, cholestanetriol, 7-keto, 25-hydroxy, and 27-hydroxycholesterol, also significantly increased in the plasma of rabbits fed the cholesterol diet. In rats the total plasma oxysterol concentration also significantly increased when feeding cholesterol but the magnitude of the increase was much less than in rabbits. 7α- and 7β-hydroxycholes-

Table 4 Selenium content and cholesterol:phospholipid ratio in erythrocytes of rabbits and rats fed basal or cholesterol-rich diets for 2 and 4 months

	2 Months				4 Months			
	Rabbits		Rats		Rabbits		Rats	
	Basal	Cholesterol	Basal	Cholesterol	Basal	Cholesterol	Basal	Cholesterol
Selenium (nmol/g Hb)	22.56 ± 0.41	15.3 ± 0.38[d]	18.72 ± 1.4	23.3 ± 2.56	21.53 ± 2.4	13.6 ± 2.3[c]	21.4 ± 1.8	24.6 ± 1.92
Cholesterol:phospholipid (mol/mol)	0.88 ± 0.15	0.87 ± 0.13	0.74 ± 0.05	0.76 ± 0.005	1.1 ± 0.1	1.86 ± 0.22[c]	0.83 ± 0.08	0.81 ± 0.06

Results are expressed as means ± SD of six animals per group. At each feeding period the mean values with a superscript letter c or d are significantly different from their controls fed the basal diet at levels of $P < 0.01$ and $P < 0.001$, respectively.

Table 5 Total oxysterol contents (μmol/L) in plasma of rabbits and rats fed basal or cholesterol-rich diet for 4 months

Oxysterol	Rabbits		Rats		Rabbits fed cholesterol vs. rats fed cholesterol
	Basal (n = 4)	Cholesterol (n = 4)	Basal (n = 4)	Cholesterol (n = 4)	
7α	0.168 ± 0.042	0.777 ± 0.0825[d]	0.228 ± 0.055	0.795 ± 0.26[b]	NS
7β	0.198 ± 0.011	1.83 ± 0.364[d]	0.185 ± 0.049	0.41 ± 0.036[c]	<0.01
β-epoxy	0.173 ± 0.069	0.609 ± 0.045[d]	0.199 ± 0.07	0.362 ± 0.18	NS
α-epoxy	0.306 ± 0.057	0.945 ± 0.025[d]	0.25 ± 0.041	0.38 ± 0.13	<0.001
Triol	0.256 ± 0.029	0.738 ± 0.154[c]	0.136 ± 0.07	0.147 ± 0.027	<0.01
7-keto	0.360 ± 0.121	1.463 ± 0.15[d]	0.53 ± 0.085	0.554 ± 0.05	<0.001
25-OH	0.053 ± 0.009	0.20 ± 0.012[d]	ND	ND	
27-OH	0.0425 ± 0.0125	0.295 ± 0.0128[d]	0.065 ± 0.01	0.1 ± 0.007[c]	<0.001
Total oxysterol (μmol/L)	1.532 ± 0.23	6.8 ± 0.675[d]	1.58 ± 0.154	2.75 ± 0.366[c]	<0.001

Results are expressed as means ± SD of four animals per group. Mean values with a superscript letter b, c, or d are significantly different from their controls fed the basal diet at levels of $P < 0.02$, $P < 0.01$, or $P < 0.001$, respectively
ND–not detectable.
NS = not significant.

terol were the only oxysterols increased in the plasma of rats fed cholesterol.

In rabbits fed the cholesterol diet erythrocyte activity of the GSH-Px decreased compared with rabbits fed the basal diet (*Figure 1A*). This decrease, however, was only significant after 2 and 4 months on the cholesterol diet. In contrast, the erythrocytes' catalase activity significantly increased after 2 and 4 months in the rabbits fed the cholesterol diet compared with the control group (*Figure 1B*). No significant change could be observed for SOD activity between the rabbits fed cholesterol or basal diets at any time (*Figure 1C*). We did not observe any significant differences in the activities of the erythrocytes' GSH-Px (*Figure 1A*), catalase (*Figure 1B*), or SOD (*Figure 1C*) between the rats fed the cholesterol or basal diets at any time. *Figure 1A* also shows that the basal activity of GSH-Px was much higher in the erythrocytes of the control rats than in the control rabbits at all times tested.

The assay of the antioxidant enzymes in the livers also revealed that feeding the cholesterol diet to rabbits significantly decreased GSH-Px (*Figure 2A*) and catalase (*Figure 2B*) activities, but did not produce any significant change in SOD activity (*Figure 2C*). In contrast, feeding the cholesterol diet to rats significantly enhanced the activities of the liver GSH-Px (*Figure 2A*) and catalase (*Figure 2B*) enzymes. Similar to rabbits no significant change was found for the SOD activity between the rats fed the cholesterol or basal diets (*Figure 2C*). The basal activities of catalase (*Figure 2B*) and SOD (*Figure 2C*) were much higher in the livers of the control rats than in the control rabbits.

Discussion

In cholesterol-fed rabbits, hypercholesterolemia was accompanied by increased hepatic secretion of β-VLDL,[42] which could explain the increase of cholesterol, TG, and PL in their plasma. In rats the plasma TG and PL were not significantly different with either the basal or the HC diets. This indicated that VLDL secretion did not differ between rats fed either diet, but that VLDL particles were enriched with higher amounts of cholesterol in rats fed the HC diet. Cholesterol feeding increased hepatic TG synthesis,[43] which explains the accumulation of TG in the livers of rats fed the HC diet. In rabbits fed the HC diet the increased secretion of β-VLDL caused large amounts of hepatic TG to assemble in these lipoproteins. This could explain the decrease of the hepatic TG level.

The increase of plasma LPX in cholesterol-fed rabbits agrees with previous reports.[1–3] Our data also showed that feeding the HC diet to rats increased neither plasma nor liver LPX compared with the control rats and agreed with previous studies.[4,44]

The increase of plasma LPX in rabbits fed the HC diet may be due to the animals' severe hypercholesterolemia because under supraphysiologic cholesterol concentration the antioxidant protection by plasma vitamin E may be insufficient to scavenge free radicals generated within a large substrate pool. The plasma cholesterol in HC rabbits was present mainly as ester of fatty acids, of which approximately 30% were polyunsaturated fatty acids (PUFA; data not shown) and were the early and more likely target for free radical oxidation.[45] Our data further indicated that the nearly twofold increase of plasma cholesterol in rats fed the HC diet did not increase plasma LPX and revealed that the plasma antioxidants were capable of maintaining the LPX within the normal level of the control rats. Indeed, the concentration of plasma α-tocopherol as related to the plasma cholesterol decreased significantly in HC rabbits but remained normal in HC rats compared with their respective controls. A decreased vitamin E:cholesterol ratio was among the strong predictors for the increased susceptibility of plasma and lipoprotein lipids to oxidation in vitro.[46] This indicates that in HC rabbits, although the number of plasma lipoprotein particles increased, the amount of vitamin E per particle decreased, hence offering less antioxidant protection.

The lack of variation for the erythrocytes' antioxidant enzyme activities between the control and HC rats could reflect the absence of excessive stress in rats fed the HC diet. In fact cholesterol feeding to rats did not alter their

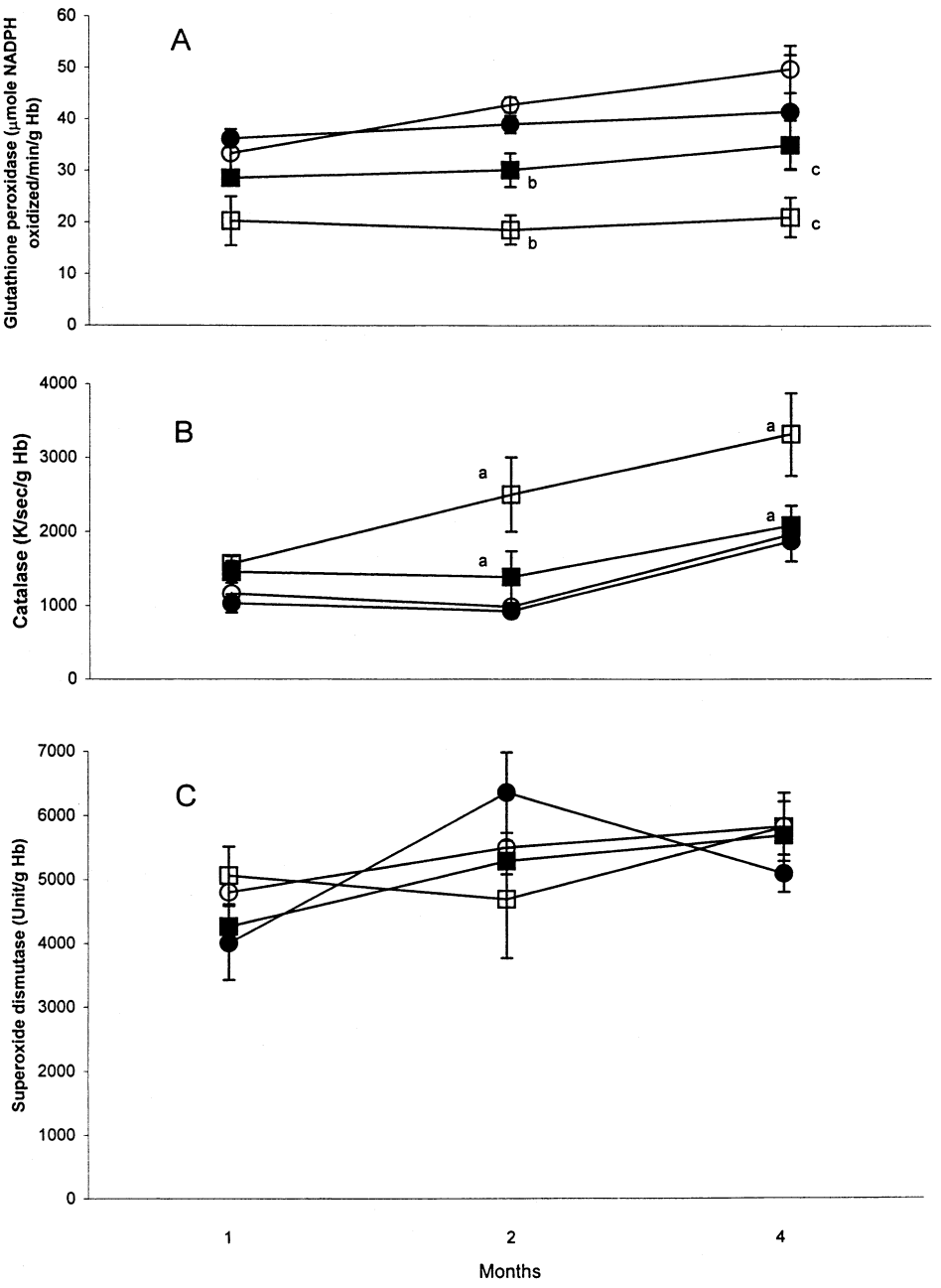

Figure 1 (A) Glutathione peroxidase (μmole NADPH oxidized/min/g Hb), (B) catalase (k/sec/g Hb), and (C) superoxide dismutase (unit/g Hb) activities in erythrocytes of rats and rabbits fed basal (● rats; ■ rabbits) or cholesterol-supplemented (○ rat; □ rabbit) diet for 1, 2, and 4 months. Results are the means of six animals per group. At each feeding period, points with a common letter (a, b, or c) are significantly different at level of $P < 0.05$, $P < 0.02$, or $P < 0.01$, respectively.

erythrocyte susceptibility to LPX or their glutathione or vitamin E levels.[47] The different plasma lipid loads achieved in rats and rabbits fed cholesterol may explain why plasma LPX and oxysterols as well as the levels of antioxidant enzymes in erythrocytes, were different. In hypercholesterolemic rabbits we found an enhanced activity of catalase whereas GSH-Px activity decreased in erythrocytes, thus agreeing with a previous report.[39] However, we did not observe any significant change in the SOD activity whereas others[39] found a significant decrease of SOD activity in the erythrocytes of rabbits fed a HC diet. We used 5% corn oil and pure cholesterol with the standard rabbit pellets whereas others[39] used 5% peanut oil and cholesterol, the purity of which was not specified. The effects of peanut oil and corn oil as well as pure cholesterol on SOD activity are not known.

We do not have an explanation for the lower selenium content in the erythrocytes of HC rabbits. Cholesterol does not seem to interfere with its absorption because plasma and liver selenium were normal in HC rabbits. Because GSH-Px is a selenoenzyme with four atoms of selenium as the active center, a lower erythrocyte selenium could affect its activity.[36] The decrease of GSH-Px activity would lower the erythrocyte capacity to deal with H_2O_2 and possibly lead to an increase in catalase activity as an adaptation process.[48] If the enhanced catalase activity is not enough to metabolize the H_2O_2, it may lead to enhanced LPX of the erythrocytes as proviously shown in cholesterol-fed rabbits.[2]

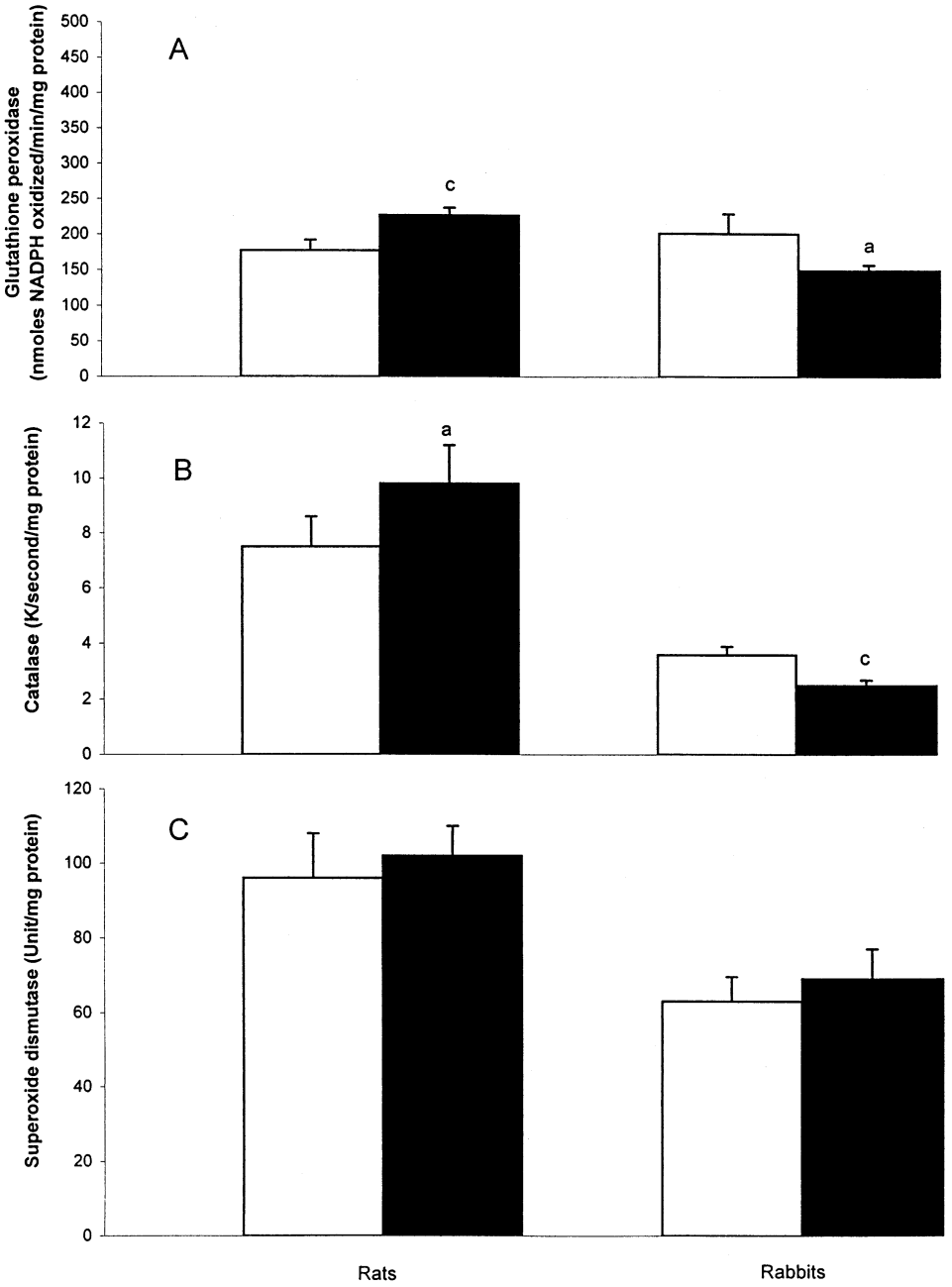

Figure 2 (A) Glutathione peroxidase (nmole NADPH oxidized/min/mg protein), (B) catalase (k/sec/mg protein), and (C) superoxide dismutase (unit/mg protein) activities in livers of rats and rabbits fed basal (□) or cholesterol-supplemented (■) diets for 4 months. Results are the means of six animals per group. Bars marked with a letter (a or c) are significantly different from their controls fed the basal diet at level of $P < 0.05$ or $P < 0.01$, respectively.

Cholesterol feeding increased liver cholesterol approximately 10-fold in both rats and rabbits, mainly as ester of fatty acids. PUFA represented 42% and 22% of these fatty acids in rat and rabbit livers, respectively (data not shown), which increase susceptibility of their liver tissues to LPX. However, the activities of GSH-Px and catalase increased in rat but decreased in rabbit livers. The basal liver SOD activity was 50% higher and catalase activity was 100% higher in control rats than in control rabbits. If rat and rabbit livers were exposed to the same level of oxygen free radicals, rat liver would be able to convert more superoxide radicals (SOR) to H_2O_2 and accumulate less SOR. The higher level of H_2O_2 can increase GSH-Px and catalase activities.[48] The relatively lower SOD activity in rabbit liver may lead to an increased level of SOR, which can inactivate GSH-Px[49] and catalase.[50] In HC rabbits the decrease of GSH-Px and catalase activities in their livers might be responsible for the increased peroxidation because it could reduce the protection of liver tissue against LPX. Consequently, in rabbits the liver could be another source of oxidized lipid such as oxysterols released into the circulation,[51] thus supporting the previous observation that rabbits fed a HC diet secrete oxidized lipoproteins.[52]

In HC rats the stimulation of the liver GSH-Px and catalase activities can protect their liver tissues against oxidation, thus preventing the accumulation of LPX prod-

ucts and oxysterols and their secretion within the lipoproteins from the liver into the circulation, as in case of HC rabbits.[52]

Contrary to our results, Tsai[53] reported a decrease of GSH-Px activity and increase of LPX in the livers of rats fed a HC diet. In the present study we used a diet that contained 5% fat plus pure cholesterol whereas Tsai used a diet that contained 15% fat plus cholesterol, the purity of which was not given. The high-fat diet with more oxidation products of cholesterol could increase LPX in the liver.[44] Tsai[53] also used cumene hydroperoxide, the specific substrate for selenium-independent GSH-Px, the minor GSH-Px activity in rat livers, whereas we used H_2O_2, the substrate for selenium-dependent GSH-Px, the major GSH-Px activity in rat livers.[54]

The liver compensates for an increase of dietary cholesterol by secreting more cholesterol into the bile.[55] Cholesterol intake enhanced 7α-hydroxylase in rat liver, which increased the pool size of 7α-hydroxycholesterol[44,56–58] and explains the increase of 7α-hydroxycholesterol in the plasma of HC rats. 7β-Hydroxycholesterol can be of nonenzymatic origin.[59] Cholesterol can be converted to 7-keto and the 7-keto to 7β-hydroxycholesterol in rat liver microsomes.[59] Under conditions where bile acid biosynthesis is stimulated, as in the case of HC rats, the blood level of 7α- and 7β-hydroxycholesterol increased.[60,61] The possible epimerization of 7α- to 7β-hydroxycholesterol in vivo has also been suggested.[61,62]

In rabbits cholesterol intake inhibited 7α-hydroxylase[63,64] but stimulated 27-hydroxylase[64] in the liver as an alternative bile acid synthesis pathway and was responsible for the increase of 27-hydroxycholesterol in the plasma of HC rabbits. It has been hypothesized that under conditions where the tissue or blood antioxidants are exhausted, the plasma OCh oxidized in the sterol β-ring (7α, 7β, α-epoxy, β-epoxy, cholestanetriol, and 7-keto) could represent past interception of vascular and tissue oxidants by cholesterol acting as an in vivo antioxidant.[61] In HC rabbits the increase of plasma and liver LPX and cholesterol could provide a higher concentration of cholesterol available for free radical auto-oxidation reaction.[65,66] In vivo formation of OCh in serum has been shown by a $^{18}O_2$ inhalation technique.[67] The significantly higher concentrations of the total as well as the individual OCh in rabbit plasma would be more cytotoxic than the OCh in rat plasma.[68]

Our data showed that feeding a HC diet to rabbits and rats increased LPX in plasma and livers of rabbits but had no effect on LPX in rats. In the plasma of rabbits, it also increased the total and individual oxysterol concentrations, which were significantly higher than in rats. This study also showed that, although cholesterol feeding to rats and rabbits produced almost the same amount of cholesterol in their livers, the liver GSH-Px and catalase activities increased significantly in rats while decreasing in rabbits. This difference may indicate that rats are capable of protecting their liver tissues from oxidation and of eliminating the possible increase of LPX and oxysterols, which can be secreted in the lipoproteins from the liver into the circulation. These different metabolic responses of rats and rabbits to a HC diet could be another factor indicating that rats are less sensitive than rabbits to the atherogenic effect of a HC diet.

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