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Hypercholesterolemia-Induced Oxidative Stress in Heart and its Prevention by Vitamin E

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Abstract. Oxygen-free radicals have been implicated in hypercholesterolemic atherosclerosis. It is possible that hypercholesterolemia produces oxidative stress in myocardium. We therefore investigated the effects of a high cholesterol diet in the absence or presence of vitamin E on serum cholesterol and lipid peroxidation product malondialdehyde (MDA), chemiluminescence (M-CL), a measure of antioxidant reserve, and activity of antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-P_x)] in cardiac muscles of rabbits. Rabbits were divided into four groups: Group I, regular rabbit chow diet; Group II, same as Group I + vitamin E; Group III, high cholesterol diet; Group IV, high cholesterol + vitamin E. The heart was removed under anesthesia at the end of 4 months on their respective diets for various biochemical measurements. Serum cholesterol in Groups III and IV increased to a similar extent. There was an increase in the levels of MDA, M-CL, GSH-P_x activity and a decrease in SOD activity in hypercholesterolemic rabbits in the absence of vitamin E. Vitamin E prevented the hypercholesterolemia-induced changes in cardiac MDA, M-CL, and GSH-P_x. These results suggest that hypercholesterolemia produces oxidative stress in the myocardium which may be due to a decrease in the antioxidant reserve, and that vitamin E is effective in preventing hypercholesterolemia-induced oxidative stress on the myocardium.

Introduction

Hypercholesterolemia is a major risk factor for coronary artery disease [1–4]. It increases the levels of lipid peroxidation product malondialdehyde (MDA) in blood and aortic tissue [5,6]. These results suggest that during hypercholesterolemia, there is an increase in the levels of oxygen-free radicals (OFRs) which could be due to increased production and/or decreased destruction. In fact, OFR-producing activity of polymorphonuclear (PMN) leukocytes (PMNL-CL) is increased in hypercholesterolemia [6]. Various factors have been implicated in the release of OFRs from PMN leukocytes during hypercholesterolemia [6]. OFRs exert their cytotoxic effects by causing peroxidation of unsaturated fatty acids of membrane phospholipids, which can result in an elevation in membrane fluidity and permeability and loss of cellular integrity [7,8]. Lipid peroxidation results in the formation of MDA. Oxidative stress is known to alter antioxidant enzymes [superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-P_x)] [9–11]. Hypercholesterolemia increases the activity of SOD, catalase, and GSH-P_x in the rabbit aorta [12].

OFRs have been implicated in the pathophysiology of numerous disease states including ischemia-reperfusion injury [13], heart failure [14], hemorrhagic shock [15], atherosclerosis [6], cardiac depression [16,17], and adult respiratory distress syndrome [18]. Vitamin E, a nonenzymatic antioxidant, is known to retard the progression of spontaneous [19] and hypercholesterolemic atherosclerosis [6]. It is possible that elevated OFRs in hypercholesterolemia may damage the myocardial cell besides affecting the coronary arteries. Oxidative stress in myocardium may also be due to a decrease in the antioxidant enzymes and nonenzymatic antioxidants. Vitamin E may be able to prevent the oxidative stress in heart during hypercholesterolemia. Therefore, we investigated the effects of hypercholesterolemia on the levels of MDA content, antioxidant enzymes, and antioxidant reserve (measure of oxidative stress) of the myocardium in rabbits. The effects of vitamin E treatment on changes in these parameters during hypercholesterolemia were also investigated.

Methods

New Zealand white rabbits weighing between 1.8 and 2 kg were divided into four groups of 7. Rabbits in Group I (control) were fed normal rabbit

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Table 1. Changes in the serum cholesterol concentrations (in mmol/L) of the four experimental groups

Groups	Time period (months)				
	0	1	2	3	4
I (Control)	1.75 ± 0.25	1.75 ± 0.25	1.75 ± 0.25	1.50 ± 0.50	1.25 ± 0.25
II (Vitamin E)	2.10 ± 0.48	1.25 ± 0.14	1.60 ± 0.10	1.50 ± 0.16	1.60 ± 0.10
III (Cholesterol)	1.33 ± 0.33	25.5 ± 6.95*ab	34.62 ± 4.62*ab	35.38 ± 3.85*abc	45.38 ± 6.92*abc
IV (Cholesterol + Vitamin E)	1.82 ± 0.24	29.0 ± 3.60*ab	38.46 ± 5.38*ab	46.92 ± 3.85*ab	57.69 ± 5.38*ab

*$p < 0.05$, comparison of the values at different times with respect to 0 month in the respective groups.
[a]$p < 0.05$, Group I vs Groups II, III, or IV.
[b]$p < 0.05$, Group II vs Groups III or IV.
[c]$p < 0.05$, Group III vs Group IV.

chow pellets (150 g/day); Group II were fed rabbit chow pellets + vitamin E (dl-α-tocopherol acetate, 1360 IU/g), 0.04 g/kg daily; Group III received cholesterol (0.5 g/kg daily) orally; and Group IV received cholesterol (0.5 g/kg daily) along with vitamin E (0.04 g/kg daily). Vitamin E was wrapped in a piece of lettuce leaf and fed orally. Cholesterol was mixed in rabbit chow diet. Serum total cholesterol was measured before and after 1,2,3, and 4 months on the experimental diets. The heart was removed under anesthesia for measurement of MDA, antioxidant enzymes, and antioxidant reserve (muscle-chemiluminescence) at the end of 4 months on their respective diets.

Serum Cholesterol

Serum total cholesterol was measured as described previously [6].

MDA (Thiobarbituric Acid-Reactive Substances)

MDA levels in cardiac tissue were estimated as thiobarbituric acid (TBA)-reactive substances by the method of Prasad et al. [6,17] and Yagi [20]. Hearts were removed, cleaned of aortic tissue, atria, and blood, and immersed in cold Hank's balanced salt solution (HBSS). Cardiac tissue was added to 10 volumes (W/V) of HBSS, and homogenized with a Polytron homogenizer at a setting of 5 for two periods of 10 seconds each at 0°–4°C. Homogenate (0.1 ml) was used for determination of MDA by the method previously described [7,17].

Cardiac Tissue Chemiluminescence (M-CL)

The homogenate as prepared above was centrifuged at 400 x *g* in a Beckman Model J2-21 refrigerated centrifuge (4°C) for 2 minutes. The supernatant was used for measurement of muscle chemiluminescence. The method for measurement of cardiac tissue chemiluminescence was similar to that reported by Prasad et al. [21]. In short, for measurement of luminol-dependent cardiac tissue M-CL, 0.8 ml of supernatant (approximately 10 mg protein) was added to a polystyrene vial containing 0.4 ml of 2×10^{-4} M luminol, which was then placed in a luminometer and incubated for 10 minutes at 37°C. Reaction was initiated by addition of 0.2 ml of tert-butyl hydroperoxide (TBHP) solution (200 mM). The final volume was 1.4 ml. When TBHP was not used, the final volume was made up with an appropriate amount of KCL-phosphate buffer. Chemiluminescence for each sample was monitored every minute for 30 minutes with or without TBHP. The area under the curve was integrated to give total CL response during the period of monitoring. The difference in the area with and without TBHP was designated as luminol-amplified CL, derived from TBHP (TBHP-derived oxygen radicals). The unit for integrated area under the curve is in mV · minute and for chemiluminescence in mV · minute/mg $protein^{-1}$.

Measurement of SOD Activity

SOD activity in supernatant was measured as previously described [12,21]. In short, 10–500 μl of supernatant was added to a sample tube containing reaction mixture which included nitroblue tetrazolium. Fifty microliters of xanthine oxidase (20 U/ml) was added to all the tubes except the reagent blank at intervals of 20 seconds to start the reaction. The final volume of the reaction mixture was 3 ml. After incubation at 25°C for 20 minutes, the reaction was terminated by adding 1 ml of 0.8 mmol/L cupric chloride. The formazan produced was measured spectrophotometrically at 560 nm.

Measurement of Catalase Activity

Catalase activity was measured as described [12,21]: 0.1 ml of supernatant was added to a cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1.0 ml of freshly prepared 20 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. The activity of catalase was expressed as $k \cdot s^{-1} \cdot$ mg $protein^{-1}$ where k is the first order rate constant.

Measurement of GSH-P_x Activity

The method for measuring GSH-P_x activity was as described [12,21]. In short, various amounts (50–200 μl) of supernatant (200–500 μg protein) were added to the tubes containing reaction mixture. The final volume of reaction mixture was 3.0 ml. The reaction was started by 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 4 minutes. GSH-P_x activity was expressed as nmoles of NADPH oxidized to $NADP^+$/minute/mg protein using an extinction coefficient (6.22×10^6) for NADPH.

Statistical Analysis

Unpaired Student's *t* test was used to analyze the results. The difference was considered significant if the *p* value was less than 0.05.

Results

Serum Cholesterol

The changes in the serum total cholesterol in the four groups are summarized in Table 1. Total cholesterol before exposure to experimental diets in Groups I, II, III, and IV were 1.75 ± 0.25, 2.10 ± 0.48, 1.33 ± 0.33, and 1.82 ± 0.24 mmol/L (SEM), respectively. Serum cholesterol levels remained unchanged in Groups I and II but increased progressively in Groups III and IV. The increases were of similar magnitude in both these groups until 2 months, but were greater in Group IV than in Group III thereafter. The levels in Groups III and IV were higher than in Groups I and II.

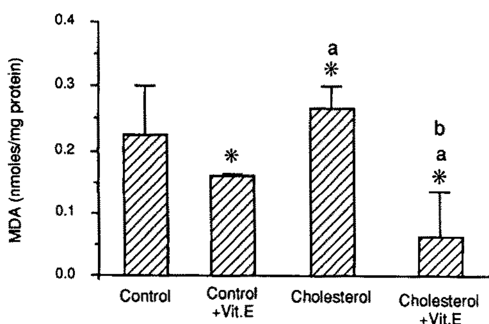

Fig. 1. Cardiac malondialdehyde (MDA) content of the four experimental groups. The results are expressed as mean ± SEM. Vit.E, Vitamin E. $^*p < 0.05$, Group I vs Groups II, III, and IV; $^ap < 0.05$, Group II vs Groups III and IV; $^bp < 0.05$, Group III vs Group IV.

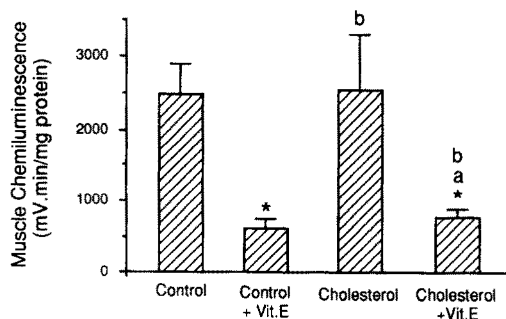

Fig. 2. Cardiac M-CL of the four experimental groups. Results are expressed as mean ± SEM. $^*p < 0.05$, Group I vs Groups II, III, and IV; $^ap < 0.05$, Group II vs Groups III and IV; $^bp < 0.05$, Group III vs Group IV.

Malondialdehyde (Thiobarbituric Acid-Reactive Substances)

MDA contents of cardiac tissue in the four groups are summarized in Figure 1. MDA was 0.225 ± 0.075 nmoles/mg protein (SEM). The content decreased significantly in groups II and IV, but increased in Group III as compared with Group I. The content in Group IV was lower than in Groups II and III. Vitamin E prevented the hypercholesterolemia-induced increase in cardiac MDA.

Antioxidant Reserve (M-CL)

The changes in the cardiac M-CL in the four experimental groups are shown in Figure 2. M-CL of control groups was 2609.33 ± 221.33 mV · minute/mg protein (SEM). M-CL decreased in Groups II and IV but increased in Group III as compared with Group I. Its values in Group IV were lower than those in Group III but higher than in Group II. These results suggest that hypercholesterolemia decreased the antioxidant reserve whereas vitamin E increased the antioxidant reserve irrespective of hypercholesterolemia.

Antioxidant Enzymes

SOD Activity. Changes in SOD activity of the four groups are summarized in Figure 3. The activity decreased in Groups II, III, and IV as compared with Group I. The activities in Groups III and IV were similar but lower than in Group II. The results suggested that SOD activity decreased in hypercholesterolemia with or without vitamin E treatment.

Catalase Activity. Changes in catalase activity of the four groups are shown in Figure 4. Catalase activity in Groups I, II, and III were similar. The activity in Group IV was lower than that in Group II.

GSH-P_x Activity. The changes in the GSH-P_x activity in the four groups are summarized in Figure 5. The activity in Groups II and III was higher than in Group I, however, it was similar in Groups I and IV. The activity in Group IV was lower than that in Groups II and III. The results indicated that hypercholesterolemia increased the GSH-P_x activity and vitamin E prevented the rise in GSH-P_x activity induced by hypercholesterolemia.

Discussion

The four experimental groups in this study were similar to those used previously in our laboratory [6] as were the amounts of cholesterol and vitamin E [6]. Various doses of vitamin E have been used in experimental animals. Viswanathan et al. [22] used 1100 U/kg of dl-L-tocophenol in hypercholesterolemic rabbits. Other investigators have used an oral dose of 600 mg [23], 1600 IU [24], and 1200–2400 IU [25] daily in healthy humans and subjects with hyperlipoproteinemia. An oral dose of 40 mg of vitamin E daily has been found to be effective in amelioration of adriamycin-induced cardiotoxicity in rabbits [26]. The dose of vitamin E used in the present study was effective in preventing hypercholesterolemic atherosclerosis in rabbits [6]. We have shown that the cholesterol diet used in the previous study produced hypercholesterolemia and that vitamin E did not lower the serum cholesterol [6].

Cardiac tissue MDA content increased in the hearts of high cholesterol-fed rabbits (Group III) and decreased in vitamin E-treated controls (Group II) and high cholesterol-fed rabbits (Group IV). The increase in MDA level in Group III suggests oxidative damage. Vitamin E not only prevented the rise in cardiac tissue MDA in cholesterol-fed rabbits but also reduced the MDA content of cardiac tissue of rabbits on the control diet. There are no available data on the effects of vitamin E on cardiac MDA level of rabbits that were fed high cholesterol diet or a control diet. However, vitamin E has been shown to reduce the MDA content of aortic tissue from hypercholesterolemic and normocholesterolemic rabbits [6]. Vitamin E is also reported to reduce plasma MDA levels in stressed-susceptible pigs [27]. Prevention of rise in MDA levels with vitamin E may be due to inhibition of lipid peroxidation. Vitamin E is a potent chain-breaking antioxidant, and its primary role is to prevent lipid peroxidative damage in the tissue [28–30]. Vitamin E traps the chain propagating peroxyl radicals and thereby reduces the length of the autooxidation chain [30]. Recently, vitamin E has

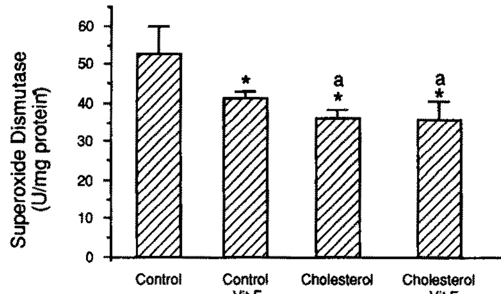

Fig. 3. Cardiac SOD activity in the four experimental groups. The results are expressed as mean ± SEM. *$p < 0.05$, Group I vs Groups II, III, and IV; [a]$p < 0.05$, Group II vs Groups III and IV.

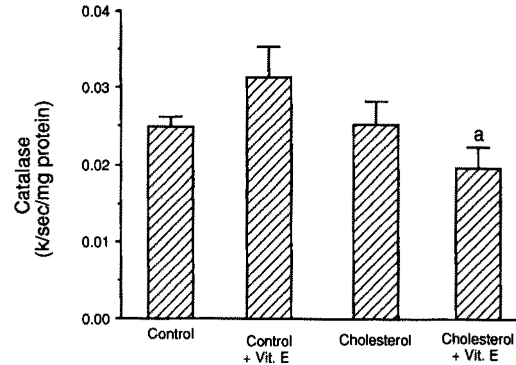

Fig. 4. Catalase activity of the cardiac muscle in the four experimental groups. The results are expressed as mean ± SEM. [a]$p < 0.05$, Group II vs Groups III and IV.

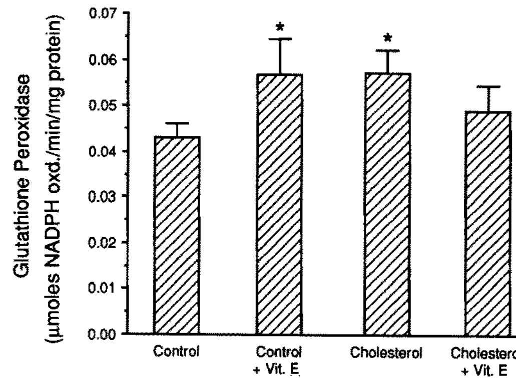

Fig. 5. GSH-P_x activity of cardiac muscle in the four experimental groups. The results are expressed as mean ± SEM. *$p < 0.05$, Group I vs Groups II, III, IV.

been reported to be a powerful scavenger of hydroxyl radicals [31].

Cardiac tissue M-CL is a measure of antioxidant reserve, an increase reflecting a decrease and a decrease reflecting an increase in this reserve [21]. An increase in the M-CL in the hypercholesterolemic rabbit indicates a decrease in the antioxidant reserve which suggests an increase in the oxidative stress. Vitamin E decreased the M-CL in normo- and hypercholesterolemic rabbits suggesting preservation of antioxidant reserve (enzymatic or nonenzymatic).

In the present study there was a decrease in the SOD activity and an increase in the GSH-P_x activity of the heart of hypercholesterolemic rabbits. Vitamin E treatment reversed the changes in GSH-P_x activity but SOD activity remained depressed. Changes in the antioxidant enzymes of the heart with hypercholesterolemia have not been reported before. However, an increase in the SOD and GSH-P_x and a decrease in the catalase activity of aortic tissue in hypercholesterolemia has been reported [32]. However, we have shown that hypercholesterolemia produced an increase in SOD, catalase, and GSH-P_x activity of aortic tissue [12]. The differences in the enzyme activity of aortic and cardiac tissue could be organ specificity. An increase in GSH-P_x activity of cardiac tissue could be due to adaptive oxidative stress. Elevation of GSH-P_x activity is commonly associated with a small increase in oxidative stress [9,10]. Shull et al. [11] have reported a reduction in SOD, catalase, and GSH-P_x activity with different forms of oxidative stress. Prevention of increase in GSH-P_x activity by vitamin E in the hypercholesterolemic rabbit could be due to a decrease in the oxidative stress as a result of antioxidant activity of vitamin E. Decrease in the SOD activity could be due to oxidative stress. However, oxidative stress would have decreased GSH-P_x also. This difference in the changes in SOD and GSH-P_x activity of the heart during hypercholesterolemia cannot be explained at present. It is possible that hypercholesterolemic stress affects these two enzymes differently.

Catalase activity was unaffected by hypercholesterolemia, however, vitamin E decreased its activity in hypercholesterolemia. Catalase plays only a minor role in the metabolism of H_2O_2 [33,34]. Studies in other tissues have also shown the importance of GSH-P_x over catalase [35,36].

The protective effect of vitamin E against hypercholesterolemia-induced oxidative stress is most probably due to its antioxidant activity. The effect is not due to lowering of blood cholesterol because blood cholesterol was not affected by vitamin E. The protective effect of vitamin E could also be due to its inhibitory effect on lipoxygenase activity [37]. Lipoxygenase increases the formation of leukotrienes and it is known that ·OH is formed during leukotriene synthesis [38].

The results of the present study show that there is an increase in the oxidative stress in the myocardium in hypercholesterolemia. The oxidative stress is associated with a decrease in the antioxidant reserve. Vitamin E, which is a scavenger of ·OH, an inhibitor of ·OH production, and a chain-breaking antioxidant, prevented the hypercholesterolemic-oxidative stress in the myocardium.

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