Selenium Supplementation and Diet Induced Hypercholesterolemia in the Rat: Changes in Lipid Levels, Malonyldialdehyde Production and the Nitric Oxide Synthase Activity

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Abstract. Male Sprague Dawley rats were divided into three groups, viz (I) Controls, (II) High fat diet (HFD) fed, (III) HFD fed+selenium supplemented. After three months of treatment, there were significant increases in serum cholesterol and triglycerides in HFD fed group as compared to control. However, in Se supplemented group, the levels of serum cholesterol and triglycerides were significantly less as compared to group II. Selenium-dependent glutathione peroxidase (GSH-Px) activity in the liver and the aorta increased significantly in HFD fed animals and also showed additional significant increase on selenium supplementation. Malonyldialdehyde (MDA) concentrations in serum, liver and aorta and the activity of nitric oxide synthase (NOS; evident from reactive nitrogen intermediates and citrulline levels) in plasma showed significant increases in HFD fed group. However, supplementation of selenium led to a significant reduction in the levels of these parameters vis-a-vis HFD fed animals except in MDA levels in the serum and the liver where this decrease was non-significant. The important finding of this study is that selenium supplementation modulates the sequences favoring pathogenesis of atherosclerosis.

Key words: High fat diet — Hypercholesterolemia — Selenium — Lipid peroxidation — Nitric oxide — Citrulline

Introduction

Hypercholesterolemia is accepted as a high risk factor for development of atherosclerosis. This is of importance since eating high fat diets (HFD) may lead to cholesterol deposition in the arterial wall (Brown and Goldstein 1984). Disease of clinical

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significance develops when influx and deposition of cholesterol exceed egress of cholesterol from arterial wall. All of these manifestations lead to vasoconstriction and arterial spasm due to the impairment of endothelium-dependent vasorelaxation (Bossaler et al. 1987; Forstermann et al. 1988).

Oxidized low density lipoproteins (LDL) are believed to be atherogenic (Steinberg et al. 1989). Oxidation of LDL is likely to proceed by mechanisms which involve free oxygen radicals generated during atherogenesis (Esterbaur et al. 1993). The concept of free radical-derived pathology is well established (Belch et al. 1989). Recent epidemiological studies have indicated that antioxidants may protect against the development of vascular diseases (Steinberg 1992). The mechanism of protection may relate to the ability of antioxidants to prevent the formation of oxidized LDL which is highly atherogenic.

An enzyme responsible for nitric oxide ($NO^{\bullet}$) production by oxidation of guanidino nitrogen atom(s) of L-arginine has recently been termed nitric oxide synthase (NOS, EC 1.14.23). NOS is of two types: constitutive and inducible. Some of the studies suggest that endothelium-dependent relaxation factor (EDRF) and constitutively produced $NO^{\bullet}$ are the same (Palmer et al. 1987), and decrease in $NO^{\bullet}$ is observed during atherogenic state whereas some of the other reports suggest its increase (Minor et al. 1990; Ohara et al. 1993).

In the present study, we examined the basic lipid levels and lipid peroxidation (MDA production) which is dependent on the FOR's generated in the system. Further, an attempt was made to evaluate the activity of NOS in HFD fed rats. To investigate the involvement of FOR's in the pathogenesis of atherosclerosis with respect to NOS activity, these studies were also done in selenium (Se, an essential trace element and an antioxidant) supplemented animals fed on HFD. Se is an essential structural component of glutathione peroxidase, GSH-Px (Wojcicki et al. 1991), which is a metabolizing enzyme for free oxygen radicals (FOR). GSH-Px activity was also measured in the present study, and was also shown to change with oxidative stress (Mantha et al. 1993; Boccio et al. 1990).

Materials and Methods

Chemicals

Cholesterol was obtained from LOBA chemicals, (Bombay, India). Sodium selenite and thiobarbituric acid were from Sigma Chemical Co. (St. Louis, USA). All other reagents and chemicals were obtained from SISCO Research Laboratories (India).

Treatment protocol

Eighteen male Sprague-Dawley rats (100–125 g) were obtained from the Central Animal House, Panjab University Chandigarh (India), acclimatized to laboratory

animal room, and then randomly divided into three equal groups: Group I, Control; Group II, High fat diet (HFD), and Group III, HFD+ Selenium. Water was given ad libitum. The treatment protocol was followed for three months.

Diet Preparation

Synthetic diet was prepared in the laboratory itself as described by Abraham et al. (1993). The compositions of the normal and the atherogenic diet is given in the Table 1. The Se supplemented group of animals received 25 μg Se (equivalent to 1.0 ppm in diet) as sodium selenite/rat/day in solution form by oral intubation. It is well established that levels exceeding 0.02 ppm are adequate Se levels in mammals, and levels beyond 2.0 ppm are considered subtoxic. We selected the above dose so as to have Se levels in excess but not toxic.

Table 1. Diet composition as according to Abraham et al (1993)

	Normal Diet (Weight %)	Atherogenic Diet (Weight %)
Corn starch	71 0	61 5
Casein	16 0	16 0
Groundnut oil	8 0	0 0
Coconut oil	0 0	15 0
Cholesterol	0 0	2 0
Sodium cholate	0 0	0 5
Salt mixture	4 0	4 0
Vitamin mixture	1 0	1 0
Potassium perchlorate	0 0	25 mg/100 g body weight/day

Before sacrifice, blood was withdrawn from overnight fasting rats from the retro orbital sinus. Plasma and serum were prepared separately according to standard procedures for further analysis. Animals were sacrificed under anesthesia and livers and aortae were removed for further analysis.

Groundnut oil and coconut oil were the two fats used for the diet preparation. The ratio of polyunsaturated and saturated fats is 0.005 for coconut oil and 3.17 for groundnut oil, as reported by Kritchevsky (1988).

Serum total cholesterol and triglycerides

Serum total cholesterol was estimated according to the method of Chiamori and Henry (1959). Triglycerides were quantitated in serum using Enzokit supplied by Ranbaxy Diagnostic Ltd. (India).

Se-dependent GSH-Px activity

Activity of GSH-Px was assayed by the coupled enzyme procedure with glutathione reductase using hydrogen peroxidase (H_2O_2) as substrate (Paglia and Valentine (1967). The enzyme activity was carried in the postmitochondrial fraction (PMF) of the liver and the aorta, and the activity was expressed as μmols of NADPH oxidized/min/mg protein.

Lipid peroxidation

Measurement of malonyldialdehyde (MDA) production (as a marker of lipid peroxidation) was done in serum samples as well as in postmitochondrial fractions of 10% homogenates of the liver and the aorta by following the modified method (Ohkawa et al. 1979) using thiobarbituric acid (TBA) reagent and measuring TBA reactive substances.

Protein estimation

Protein estimation in the serum as well as in tissue homogenates was done using the modified method of Lees and Paxman (1972).

Nitric oxide synthase (NOS) activity

$NO^{\bullet}$ in terms of nitrite, a stable metabolic product of $NO^{\bullet}$, was assayed in the plasma using Griess reaction, and citrulline (a stable by-product of the NOS-catalyzed reaction) was also measured in the plasma according to the classical spectrophotometric assays as per standard protocol (Raddassi et al. 1994).

a) *$NO^{\bullet}$ levels*: $NO^{\bullet}$ was assessed in the plasma by measuring nitrite (a stable metabolic product of $NO^{\bullet}$) using Griess reaction. In brief, Griess reagent mixture of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H_2O and 1% sulfanilamide in 2.5% orthophosphoric acid (1:1 v/v) were mixed with equal volume of diluted plasma, and incubated for 30 min in dark. Absorbance was measured at 546 nm. The amount of nitrite was calculated using $NaNO_2$ standard curve.

b) *Citrulline levels*: The citrulline assay based on its reaction with diacetyl monoxime, was performed as follows. Plasma was incubated with urease (21 mmol/l) for 1 h at 37°C. Proteins were removed by the addition of 100 μl of $ZnSO_4$ (30%) followed by centrifugation. Ten μl of supernatant was diluted to 500 μl with 0.1 N HCl, and 1.5 ml of freshly prepared chromogenic solution was added and vortexed. Then mixture was boiled in water bath for 5 min at 100°C in stoppered tubes, and absorbance was measured at 530 nm. The amount of citrulline was calculated from citrulline standard curve.

Statistics

Data were expressed as mean ± S.E.M. Differences between groups were tested using Student's t'test for unpaired values.

Results

Rats fed HFD showed highly significant levels of total cholesterol ($p < 0.001$) as well as triglycerides ($p < 0.001$) in the serum when compared to the control rats (Table 2). However, following the administration of selenium along with HFD diet feeding, there were significantly decreased levels of total cholesterol ($p < 0.001$) as well as triglycerides ($p < 0.001$) as compared to animals fed HFD only (Table 2). GSH-Px activity was significantly higher both in the liver ($p < 0.05$) and the aorta ($p < 0.05$) of HFD fed animals. This activity was even higher ($p < 0.05$) in Se supplemented HFD fed animals as compared to group II animals (Table 2).

Table 2. Lipid profile, MDA levels, nitrite ($NO^{\cdot}$) levels, citrulline levels, and GSH-Px activity in rats after HFD and selenium treatment

	Groups		
Parameter	Control	HFD group	HFD+Se
Serum cholesterol (mmol/l)	1 44 ± 0 19	23 91 ± 0 98***	14 92 ± 1 39***
Serum triglycerides (mmol/l)	0 78 ± 0 08	1 74 ± 0 11***	0 87 ± 0 09***
Glutathione peroxidase (μmol of NADPH oxidized/min/mg protein)			
Liver	441 66 ± 34 17	549 66 ± 19 22*	614 33 ± 9 17*
Aorta	196 00 ± 19 12	279 33 ± 19 41*	481 00 ± 89 00*
MDA (nmol/mg protein)			
Serum	0 39 ± 0 02	0 57 ± 0 07*	0 44 ± 0 03
Liver	0 76 ± 0 12	1 25 ± 0 20*	0 96 ± 0 05
Aorta	0 43 ± 0 05	1 05 ± 0 09***	0 62 ± 0 02***
$NO^{\cdot}$ (nmol/ml)	4 1 ± 0 8	68 8 ± 1 9***	22 3 ± 8 2***
Citrulline (nmol/l)	1743 7 ± 240 4	4114 3 ± 218 2***	2560 0 ± 194 1***

The data represent mean ± S E M for 6 animals in each group $^{*}p < 0\ 05$, $^{***}p < 0\ 001$

There was a significant increase in the production of MDA in the serum ($p < 0.05$), the liver ($p < 0.05$) as well as the aorta ($p < 0.001$) of high fat diet fed animals, thus suggesting increased lipid peroxidation in animals suffering from hyperlipidemic condition as compared to control animals. However, MDA decreased

significantly in the aorta ($p < 0.001$) but nonsignificantly in the serum and the liver of animals supplemented with Se as compared to animals of group II (Table 2)

Nitrite levels in the plasma were seen to increase significantly ($p < 0.001$) upon HFD feeding (Table 2) as compared to control group However, these levels decreased significantly when Se was supplemented along with HFD (Table 2) Similar was the trend for plasma citrulline values where again its levels increased very significantly ($p < 0.001$) in HFD fed group and decreased ($p < 0.001$) in HFD+Se fed group (Table 2) These results suggest increased NOS activity upon HFD feeding Administration of Se along with HFD showed a significant decrease in both $NO^{\cdot}$ and citrulline levels in group III

Discussion

The role of lipid peroxides, subject of considerable current interest, is still not explained well in the genesis of atherosclerosis and cardiac diseases In the present study, the levels of lipid peroxides (MDA) increased very significantly upon HFD administration Lipid peroxidation is believed to be involved in the oxidative modification of LDL resulting ultimately in the formation of atherogenic plaques and fatty deposits at the injured site on the intima Levels of GSH-Px were also higher in the aorta and the liver of HFD fed animals These results are supported by the earlier reported findings of Boccio et al (1990) and Mantha et al (1993) Increased GSH-Px activity in HFD fed rats could be due to oxidative stress, as has also been reported earlier (Mantha et al 1993)

Moreover, the role of $NO^{\cdot}$ in cardiovascular pathology is still a point of discussion regarding its production during atherogenic condition In the present study we observed an enhanced NOS activity in rats fed HFD This is in agreement with some of the recent reports (Minor et al 1990, Ohara et al 1993) which have been carried out in the aorta of rabbits and showed increased $NO^{\cdot}$ levels

However, these results are in contradiction with some previously reported studies (Ignarro et al 1990 Moncada et al 1991) which showed decreased levels of $NO^{\cdot}$ during cholesterol induced atherogenesis It is important to mention here that these studies reported in the literature were mostly carried out on aortic rings, where NOS activity (as $NO^{\cdot}$ levels) had been measured indirectly in terms of vascular relaxation In the present study however, NOS activity was measured directly by biochemical assessment of $NO^{\cdot}$ and citrulline using well known procedures reported earlier (Raddassi et al 1994) Based on the reports that EDRF and NOS catalyzed products are not related to each other (Myers et al 1990) reflection of NOS activity as direct assessment as $NO^{\cdot}$ levels is suggested to be more plausible than its indirect measurement as vascular relaxation Hence, this study and a few previously reported findings, strongly suggest that the enzymatic process responsible

for NO• production is unregulated, rather than impaired, as a result of cholesterol feeding.

In contrast to the increased basal lipid levels, lipid peroxidation as well as increased NOS activity during atherogenic condition, we observed that when Se was supplemented along with HFD, the above levels decreased towards control. Reduction in MDA concentration following Se supplementation along with HFD suggests that Se, being an antioxidant, is an effective means of reducing lipid peroxidation which is one of the factors initiating atherosclerotic progression. The present study and the earlier reports (Wojcicki et al. 1991) indicate the protective effect of Se on the events leading to atherogenicity. GSH-Px activity is further enhanced by Se supplementation, and this is in agreement with the well known fact that Se induces GSH-Px (Sunde et al. 1987). The oxidative stress of HFD feeding makes it even more pronounced.

In conclusion, feeding of high fat diet upregulates the lipid peroxidation process and NOS activity. However, selenium administration along with HFD antagonizes the said increase

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