BioMetals (2006) 19:493–501
DOI 10.1007/s10534-005-5393-z

Hypercholesterolemia and LDL receptor mRNA expression: modulation by selenium supplementation

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Received 8 September 2005; Accepted 22 November 2005

Key words: cholesterol, hypercholesterolemia, LDL-R, selenium

Abstract

Selenium (Se) status has been associated with cardiovascular disorders. Present study was aimed to elucidate the protective role of Se supplementation on LDL receptor (LDL-R) activity as well as mRNA expression during experimental hypercholesterolemia in SD male rats. Animals were fed 0.2 and 1 ppm Se supplemented control diet as well as 2% cholesterol supplemented diet for 3 months. LDL-R activity was measured *in-vivo* by injecting radiolabeled LDL to rats and decrease in counts per minute with time was taken as a measure of LDL clearance and in turn LDL-R activity. LDL-R mRNA expression was studied by RT-PCR. LDL-R activity and mRNA expression decreased significantly on 2% cholesterol supplemented diet feeding. On 1 ppm Se supplementation LDL-R activity as well as mRNA expression increased significantly. Present results demonstrate that Se supplementation upto 1 ppm is responsible for up regulation of LDL-R activity as well as mRNA expression, during hypercholesterolemia. These findings highlight the therapeutic potential of Se supplementation in lipid metabolism.

Introduction

Hypercholesterolemia is associated with an elevation of plasma low-density lipoproteins (LDL). LDL is the major carrier of cholesterol in the blood and is most significantly associated with atherosclerotic plaque formation (Gouni-Berthold & Sachinidis 2004). Basically, low-density lipoprotein receptor (LDL-R) mediates the removal of LDL from circulation by binding to apolipoproteins (Ouguerram *et al.* 2004). On feeding cholesterol supplemented diet the LDL-R deficient subjects accumulate much higher cholesterol levels than normal subjects and in turn develop atherosclerosis (Ishibashi *et al.* 1993).

Involvement of thyroid hormones in cardiovascular system is also an established fact now (Pingitore *et al.* 2005). Both hypothyroidism and hyperthyroidism are related to cardiovascular risk (Canturk *et al.* 2003). Thyroid hormone replacement therapy improved the cardiac functioning in several patients (Siegmund *et al.* 2004). Triiodothyronine (T_3) is directly involved in the regulation of LDL-R gene expression. Normally, thyroid is the unique source of thyroxine (T_4), but it secretes only 20% of the whole T_3 in the body (Engler & Burger 1984). Major amount of T_3 is produced from T_4 by 5′-deiodination in peripheral tissues (Vander Geyten *et al.* 2005). This reaction is catalyzed by type-I 5′-iodothyronine deiodinase (5′-DI). Behne *et al.* (1990) have concluded that type-I 5′-iodothyronine deiodinase is a selenoprotein and Se is situated at its active site as selenocysteine.

Several studies related hypercholesterolemia with Se deficiency (Huang *et al.* 2002; Lee *et al.* 2003). Vijaya *et al.* (2000) reported that Se deficiency is associated with cardiomyopathy resulting in congestive heart failure. Nassir *et al.* (1997) demonstrated that Se deficiency lead to increased HMG-CoA reductase activity (rate controlling

enzyme in the cholesterol biosynthesis) that inturn resulted in increased endogenous cholesterol synthesis. On the contrary, Se supplementation proved beneficial during myocardial ischemia (Poltronieri *et al.* 1992). Hence in view of all the above stated findings, the present study is aimed to understand the effect of Se supplementation on LDL-R activity as well as mRNA expression during experimental hypercholesterolemia in Sprauge–Dawley male rats.

Materials and methods

Animals and study design

Male Sprauge–Dawley rats (100 g-body weight) were used in the present study. Animals were obtained from the Central Animal House, Panjab University, Chandigarh, India. Animals were acclimatized to the laboratory animal room conditions and divided into four groups viz.: Group Ia (0.2 ppm Se supplemented diet fed control); Group Ib (0.2 ppm Se and 2% cholesterol supplemented diet fed group); Group IIa (1 ppm Se supplemented diet fed control); Group IIb (1 ppm Se and 2% cholesterol supplemented diet fed group). Treatment protocol was for 3 months.

Diet preparation

Yeast based synthetic control diet was prepared in the laboratory itself as per composition given by Burk (1987). It contained torula yeast (inactivated) 30%, sucrose 56.99%, corn oil 6.67%, mineral mix 5%, vitamin mix 1%, dl-methionine 0.3% and vitamin-E 0.04%. It is well established that 0.2 ppm of Se is considered to be the adequate dose and levels beyond 2 ppm are considered subtoxic (Kang *et al.* 1998).

After completion of diet feeding schedule, the rats were fasted for 10 h, anesthetized and then exsanguinated. Blood samples and liver tissues were collected from all the groups. Liver was snap frozen in liquid nitrogen. Serum total cholesterol and LDL levels were estimated by enzymatic colorimetric test kits obtained from E. MERCK Diagnostic (Germany). Various parameters were carried out as detailed below.

Selenium levels

Se levels in serum and liver were estimated by fluorimetric method (Hasunuma *et al.* 1982). The assay is based on the principle that Se content in samples on acid digestion is converted to selenous acid. The reaction between selenous acid and aromatic-*o*-diamines such as 2,3-diamino-naphthalene (DAN) leads to the formation of 4,5-benzopiazselenol, which displays brilliant lime-green fluorescence when excited at 366 nm in cyclohexane. Fluorescence emission in extracted cyclohexane was read on fluorescence spectrophotometer using 366 nm as excitation wavelength and 520 nm as emission wavelength.

Se-dependent GSH-Px activity

Glutathione peroxidase (GSH-Px) enzyme activity was measured by following the method of Paglia & Valentine (1967). The assay was carried out in the post-mitochondrial fraction (PMF) of liver as already published by us (Dhingra *et al.* 2003). Oxidation of NADPH was recorded at 340 nm in spectrophotometer (UV 160A, Shimadzu). The enzyme activity was expressed as μ mol of NADPH oxidized/min/mg protein. The total protein estimation was done by the method of Lowry *et al.* (1951).

Thyroid hormone (T_3 and T_4) level

Thyroid hormone (T_3 and T_4) level was estimated in serum by radioimmunoassay (RIA) kits procured from BARC, Mumbai (Cat. No. RIAK-4/4A and RIAK-5/5A for T_3 and T_4, respectively).

Type-I iodothyronine deiodinase (5′-DI) activity

Hepatic 5′-DI activity was estimated by following the modified method of Behne *et al.* (1990) based on RIA.

Low density lipoprotein receptor activity

Low density lipoprotein receptor (LDL-R) activity was estimated *in vivo* by following the method of Brown and Goldstein (1984) and as per methodology already used by us (Kang *et al.* 2000). LDL isolated from overnight fasting human plasma using a single vertical spin density gradient

ultracentrifugation (Chung *et al.* 1986), was radiolabeled with Na[^{131}I] using chloramine-T (Salahuddin & Singh 1983). Radiolabeled protein and unbound radioiodide were separated by gel filtration through sephadex G-25 column. The sterilized radiolabeled LDL was injected to rats of different groups. One ml of blood was taken 2 h after injection to measure the counts of radiolabeled LDL, and taken as counts at zero time interval or initial counts. Subsequent counts were taken at 24, 48, 72, 96 and 120 h after injection. Decrease in the counts per minute (cpm) at increasing time interval was considered as a measure of clearance of LDL from animal blood and in turn as indirect measurement of the LDL-R activity.

mRNA expression analysis

The mRNA expression for LDL-R and 5′-DI was done in liver using RT-PCR kit from QIAGEN.

RNA isolation

Total RNA from liver was extracted using TRI REAGENT (Molecular Research Centre, Inc. Ohio). The integrity and size distribution (quality) of RNA was checked by formaldehyde agarose gel electrophoresis.

RT-PCR

2 μg of total RNA template from different groups after treatment with DNase-I (Ambion) was used in RT-PCR, to the reaction mixture added10 μl of 5X QIAGEN One Step RT-PCR buffer (2.5 mM $MgCl_2$ as final concentration),2 μl of dNTP mix (10 mM of each dNTP),5 μl of each forward and reverse gene specific primers (from10 μM stock), 2 μl QIAGEN One Step RT-PCR enzyme mix,1 μl RNase inhibitor (1U/μl) and finally PCR grade water (provided in the kit) to make total volume 50 μl. Mixed it gently by vortex and centrifuged it briefly. The PCR was performed in the thermal cycler (Techne Ltd. England) using following conditions: the RT reaction was performed at 50 °C for 50 min, initial PCR activation was done at 95 °C for 15 min, followed by 35 cycles of 94 °C (denaturation) for 45 s, 58.8 °C (annealing) for 45 s, 72 °C (extension) for 1 min. Finally, incubated at 72 °C for 10 min to extend any incomplete single strands.

Optimal oligonucleotide primer pairs for RT-PCR were selected with the aid of software Gene Runner. The primer sequence (5′–3′) for rat LDL-R gene coding (+) strand was ACCGCC ATGAGGTACGTAAG, noncoding (-) strand was GGGTCTGGACCCTTTCTCTC, for 5′-DI gene coding (+) strand was TCTGGGATTTCATT-CAAGGC, noncoding strand was TAGAGCC TCTCAGGCAGAGC and for rat β-actin gene coding (+) strand was AGAGCTATGAGCT GCCTGAC, and the noncoding (-) strand was CTGCATCCTGTCAGCCTACG. The length of RT-PCR products was 341 bp, 346 and 236 bp, respectively for LDL-R, 5′-DI and β-actin. PCR products were analyzed by 1.5% agarose gel electrophoresis.5 μl of PCR product was used from each tube. Densitometric analysis of the bands was done by UviBandMap software (Uvitech, England).

Above described RT-PCRs for LDL-R, 5′-DI and β-actin were determined on the basis of a series of experiments to test the reaction conditions, such as amount of RNA linearity, cycle linearity and primer concentrations etc. The final RT-PCR conditions were shown to be in the linear range of amplification for these genes.

Statistical analysis

Data is expressed as mean $\pm$SD. Difference between different groups was tested using student's *t*-test for unpaired values.

Results

Selenium levels

Se levels in serum and liver decreased significantly ($p < 0.001$) in 2% cholesterol supplemented diet fed groups in comparison to respective controls in both 0.2 ppm as well as 1 ppm Se supplemented groups. Whereas significant increase ($p < 0.001$) in the level was observed on 1 ppm Se supplementation (IIa and IIb) in comparison to respective 0.2 ppm Se supplemented groups i.e. Ia and Ib (Figure 1).

Glutathione peroxidase activity

Glutathione peroxidase (GSH-Px) activity in liver increased significantly ($p < 0.001$) on 2% cholesterol supplemented diet feeding in comparison to respective control groups. On 1 ppm Se supplementation

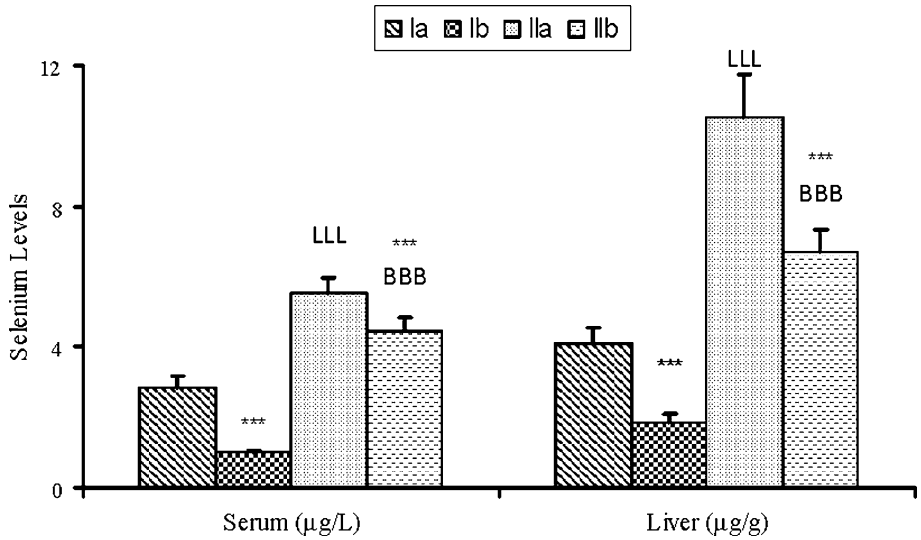

Figure 1. Selenium levels in serum and liver in different groups: (Ia) (0.2 ppm Se supplemented control), (Ib) (0.2 ppm Se and 2% cholesterol supplemented), (IIa) (1 ppm Se supplemented control), (IIb) (1 ppm Se and 2% cholesterol supplemented) after 3 months of diet feeding schedule. Data is represented as mean ± SD from 6 observations. $^{***}p < 0.001$ represent comparison between control and 2% cholesterol supplemented groups; $^{LLL}p < 0.001$ comparison between group (Ia) and (IIa); $^{BBB}p < 0.001$ comparison between group (Ib) and (IIb).

Table 1. T_3, T_4, 5′-DI and GSH-Px levels after 3 months of respective diet feeding schedule.

0.2 ppm Se supplemented group		1 ppm Se supplemented group	
Control Ia	2% cholesterol supplemented Ib	Control IIa	2% cholesterol supplemented Ib
T_3(ng/ml)			
40.08 ± 3.04	$16.49 \pm 2.97^{***}$	71.02 ± 3.69^{LLL}	$57.84 \pm 3.00^{***BB}$
T_4(µg/ml)			
3.77 ± 0.20	$5.49 \pm 0.37^{***}$	2.08 ± 0.16^{LLL}	$2.44 \pm 0.13^{***BB}$
5′-DI in liver (pmol of T_3 liberated/min/mg of protein)			
9.03 ± 0.87	$5.21 \pm 0.48^{***}$	23.61 ± 2.24^{LLL}	$17.44 \pm 1.52^{***\ BBB}$
GSH-Px levels (µmol of NADPH oxidized /min/mg protein)			
407.14 ± 48.14	$692.11 \pm 30.15^{***}$	789.23 ± 46.92^{LLL}	$982.34 \pm 71.10^{***\ BBB}$

Data is represented as mean ± SD from 6 observations. $^{***}p < 0.001$ represent comparison between control and 2% cholesterol supplemented groups; $^{LLL}p < 0.001$ comparison between group (Ia) and (IIa); $^{BB}p < 0.01$, $^{BBB}p < 0.001$ comparison between group (Ib) and (IIb).

also the GSH-Px levels increased significantly ($p < 0.001$) in comparison to respective 0.2 ppm Se supplemented groups (Table 1).

Total cholesterol and LDL level

In both the 0.2 and 1 ppm Se fed groups on 2% cholesterol supplementation significant increase ($p < 0.001$) in total cholesterol and LDL–cholesterol concentration was observed in comparison to respective control groups. On 1 ppm Se supplementation the lipid levels decreased significantly ($p < 0.001$) in comparison to respective 0.2 ppm Se fed groups (Ia and Ib) (Figure 2).

Thyroid hormone (T_3 and T_4) levels

Levels of T_3 decreased and T_4 increased significantly ($p < 0.001$) on 2% cholesterol supplemented diet feeding. On 1 ppm Se supplementation the T_3 level increased and T_4 level decreased significantly (Table 1).

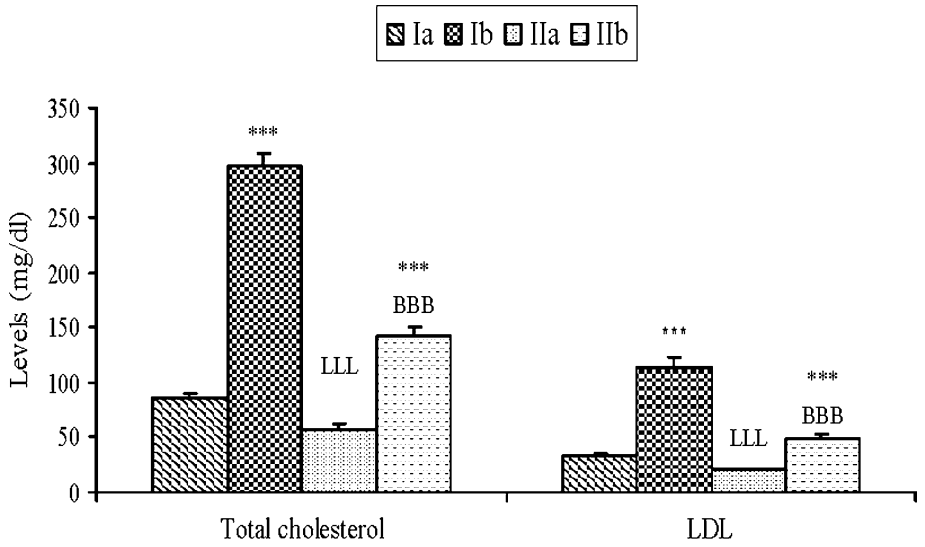

Figure 2. Total cholesterol and LDL levels in serum in different groups: (Ia) (0.2 ppm Se supplemented control), (Ib) (0.2 ppm Se and 2% cholesterol supplemented), (IIa) (1 ppm Se supplemented control), (IIb) (1 ppm Se and 2% cholesterol supplemented) after 3 months of diet feeding schedule. Data is represented as mean ± SD from 6 observations. $^{***}p < 0.001$ represent comparison between control and 2% cholesterol supplemented groups; $^{LLL}p < 0.001$ comparison between group (Ia) and (IIa); $^{BBB}p < 0.001$ comparison between group (Ib) and (IIb).

Type-I iodothyronine deiodinase (5′-DI) activity and mRNA expression

Hepatic 5′-DI activity decreased significantly ($p < 0.001$) in 2% cholesterol supplemented diet fed animals (Ib and IIb) in comparison to respective controls. On 1 ppm Se supplementation the activity increased significantly ($p < 0.001$) in comparison to 0.2 ppm Se fed groups (Table 1).

RT-PCR products of expected size i.e. 346 and 236 bp were obtained for 5′-DI and β-actin, respectively. On 2% cholesterol supplementation the mRNA expression decreased ($p < 0.001$) in liver in comparison to respective control groups. On 1 ppm Se supplementation the mRNA expression increased significantly ($p < 0.001$) in comparison to 0.2 ppm Se fed groups (Figure 4).

LDL-R activity and mRNA expression

Remaining counts of radiolabeled LDL in blood after 120 h were higher in 2% cholesterol supplemented diet fed groups in comparison to respective control groups, hence the LDL-R activity decreased on cholesterol feeding. On 1 ppm Se supplementation LDL-R activity increased significantly in comparison to 0.2 ppm Se supplemented groups i.e. the remaining counts in blood decreased on 1 ppm Se supplementation. (Figure 3).

mRNA expression followed the same trend as it was observed in case of LDL-R activity i.e. the expression decreased significantly ($p < 0.01$) on 2% cholesterol supplemented diet feeding in comparison to respective control groups. On 1 ppm Se supplementation, significant increase ($p < 0.001$) in mRNA expression was observed in comparison to 0.2 ppm Se fed groups (Figure 4).

Discussion

A link between Se status and cardiovascular disorders was originally proposed based upon the etiological studies in which the Se content of crops and drinking water was related to its concentration in blood and to the regional cardiovascular disease mortality rates (Schamberger *et al.* 1979). Salonen *et al.* (1991) reported that the serum Se concentration is related to the progression of carotid artery atherosclerosis. In the current study, 2% cholesterol supplemented diet feeding leads to low Se levels and increased GSH-Px levels (Figure 1 and Table 1). This can be attributed to the increased oxidative stress associated with hypercholesterolemia that inturn induces GSH-Px activity, as already established in author's lab (Kang *et al.* 1998). This induction of GSH-Px (Se

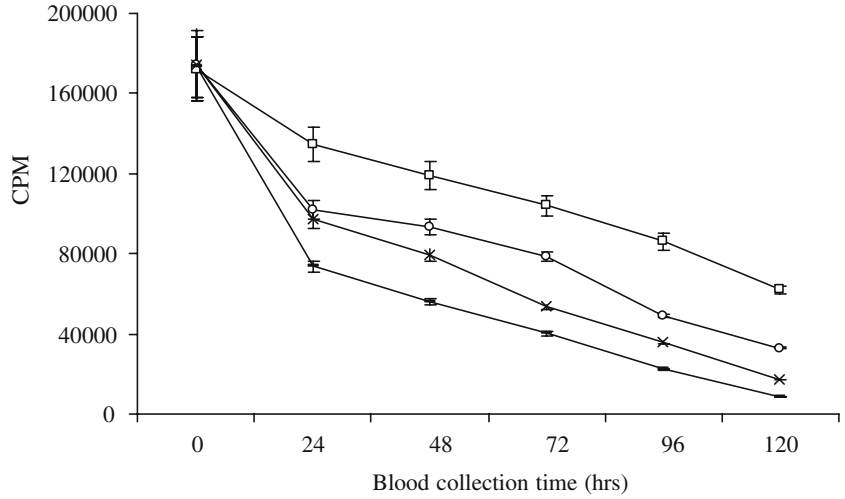

Figure 3. LDL-R activity (*in-vivo*) in different groups. (o) Ia-0.2 ppm Se supplemented control group, ( ) Ib-0.2 ppm Se and 2% cholesterol supplemented diet fed group, (−) IIa-1 ppm Se supplemented control group, (×) IIb-1 ppm Se and 2% cholesterol supplemented diet fed group. Radiolabeled LDL was injected to the rats, decrease in cpm in blood with time was taken as a measure of clearance of LDL from animal blood and in turn the LDL-R activity.

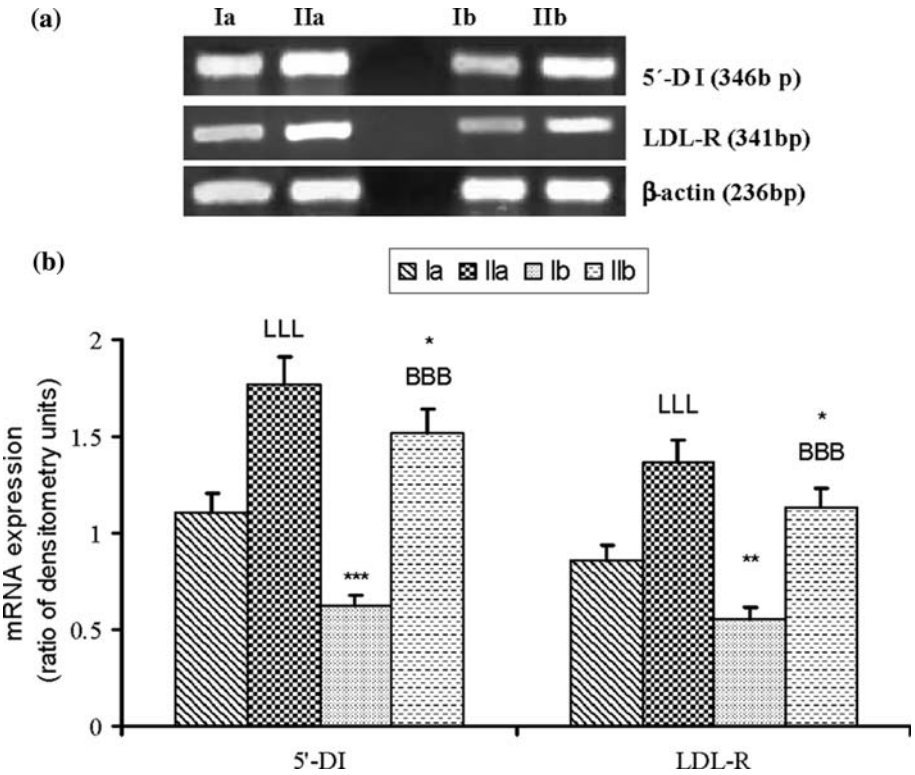

Figure 4. Hepatic 5′-DI and LDL-R mRNA analysis in different groups: (Ia) (0.2 ppm Se supplemented control), (IIa) (1 ppm Se supplemented control), (Ib) (0.2 ppm Se and 2% cholesterol supplemented), (IIb) (1 ppm Se and 2% cholesterol supplemented) after 3 months of diet feeding schedule. (a) mRNA expression by RT-PCR.β-actin was co-amplified as an internal control, (b) Bands were quantified by densitometric analysis. The relative levels of mRNA expression for 5′-DI and LDL-R were normalized to β-actin. Data is expressed as mean ± SD from 4 observations. $^{*}p < 0.05'$, $^{**}p < 0.01$, $^{***}p < 0.001$ represent comparison between control and 2% cholesterol supplemented groups; $^{LLL}p < 0.001$ comparison between group (Ia) and (IIa); $^{BBB}p < 0.001$ comparison between group (Ib) and (IIb).

containing enzyme) might be responsible for more Se consumption and inturn low Se levels. Further, low Se levels are associated with increased platelet aggregation and thromboxane A_2 production along with decreased prostacyclin production, all of which may be linked with cardiovascular diseases (Huang *et al.* 2002).

In the present study, total cholesterol and LDL levels decreased significantly on 1 ppm Se supplementation in groups IIa and IIb. Wojcicki *et al.* (1991) reported an increase in HDL cholesterol fraction on Se supplementation. HDL cholesterol fraction may down regulate the total cholesterol via reverse cholesterol transport to the liver i.e. HDL fraction increases the cholesterol elimination from tissues including smooth muscle cells in the aorta wall and facilitate the cholesterol transport to the liver, thus preventing its deposition and formation of atheromatous plaque (Marks 1979). Further, upregulation of LDL receptor expression as observed in the present results on Se supplementation might have offered more receptor sites for LDL binding and its clearance from the circulation.

Type-I 5′-iodothyronine deiodinase (5′-DI) is one of the principal enzymes involved in intrathyroidal and peripheral metabolism of thyroid hormones (Vander Geyten *et al.* 2005) as it converts T_4 to T_3. In the present study, on 2% cholesterol supplemented diet feeding, 5′-DI activity as well as mRNA expression decreased in liver. This could be due to the fact that hypercholesterolemia, which is associated with Se deficiency, might have depleted the Se pool required for normal 5′-DI (selenoprotein) expression. Further, on 1 ppm Se supplementation a significant increase in 5′-DI expression in liver was observed. Being a selenoprotein, the 5′-DI activity as well as mRNA expression might have increased on Se supplementation. Gross *et al.* (1995) reported in their cell culture experiments in porcine kidney cells that 5′-DI activity as well as mRNA levels rapidly increased with the increase in Se concentration. Depalo *et al.* (1994) in SD male rats reported that Se supplementation to Se deficient animals resulted in significant increase in 5′-DI activity in liver and kidney.

Lipid abnormalities may attribute to the impaired thyroid function (Canturk *et al.* 2003). In the present studies, on 2% cholesterol supplemented diet feeding, T_3 levels decreased and T_4 levels increased significantly. This could be owing to the decreased conversion of T_4 to T_3 due to decreased 5′-DI expression on cholesterol supplemented diet feeding. So the present study clearly indicates a strong link between hypercholesterolemia and hypothyroidism (Glueck *et al.* 1991; Wojcicki *et al.* 1991). Sundaram *et al.* (1997) have shown that LDL is more susceptible to oxidation in patients with hypothyroidism, this oxidized LDL is not taken up by LDL receptors, it accumulates in the body and inturn leads to hypercholesterolemia. On 1 ppm Se supplementation the T_3 level significantly increased and T_4 level decreased, this could be due to the increased 5′-DI expression on Se supplementation that further might have upregulated the T_4 to T_3 conversion.

Thyroid hormone replacement therapy in hypercholesterolemia resulted in marked decrease in total cholesterol and LDL cholesterol levels (Arem & Patsch 1990). Present results are also in agreement to this fact, because in group IIa and IIb animals as the T_3 levels increased total cholesterol as well as LDL concentrations decreased significantly. Basically T_3 is directly involved in the regulation of LDL-R expression via modulation of SREBP-2 (sterol regulatory element-binding protein-2) gene expression. SREBP-2 is a major transcriptional regulator of cholesterol uptake through LDL-R (Shin & Osborne 2003). Present studies demonstrated that on 1 ppm Se supplementation the LDL-R activity as well as mRNA expression is significantly increased in comparison to 0.2 ppm Se fed animals. This increase in LDL-R activity as well as expression could be due the increased T_3 level observed on 1 ppm Se supplementation through upregulation of 5′-DI expression. Also as we have observed that on 1 ppm Se supplementation total cholesterol as well as LDL level decreased, this decreased intracellular cholesterol level might have upregulated the LDL-R expression through feedback signaling pathway. Goldstein and Brown (1990) have suggested that LDL-R expression is regulated predominantly through cholesterol negative feedback pathway. On feeding 2% cholesterol supplemented diet to the animals, LDL-R activity and mRNA expression decreased in both 0.2 ppm as well as 1 ppm Se supplemented groups, so exogenous cholesterol given through diet is being used in the signaling pathway and probably it is suppressing the transcription of LDL-R through feedback mechanism.

Present study provides the first evidence that Se supplementation upto 1 ppm is responsible for up regulation of LDL-R activity as well as mRNA expression, during hypercholesterolemia. This might be owing to the upregulation of Se dependent 5′-DI expression, leading to increased T_3 levels. These findings highlight the therapeutic potential of Se supplementation in lipid metabolism through its dependent enzyme, 5′-DI. However, this interrelationship between Se status and LDL-R gene expression warrants further investigation to decide the precise mechanism of cholesterol metabolism through the effect of Se status on the LDL-R gene expression.

Acknowledgements

Authors acknowledge the financial support given by the Department of Atomic Energy, Government of India, Mumbai (India).

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