Experimental
2.1.1. Chemicals

The chemicals were obtained from various sources listed below.

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<th>Chemical</th>
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TPCK - Sigma Chemicals, USA
Triton X 100 - SRL, India
Tween-20 - Sigma Chemicals, USA

Anti-rat hepatic cytosolic CEH antisurum was a kind gift from Dr. Shobha Ghosh, Department of Biochemistry, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia, USA. Anti-rat pancreatic CEH IgG was a kind gift from Dr. Linda L. Gallo, Department of Biochemistry, George Washington University, Washington D.C. USA.

All other chemicals and reagents used were of analytical grade.

2.1.2. Animals

Male albino rats, weighing about 175-200 g were purchased from Central Animal House, J.N. Medical College, A.M.U., Aligarh. Rats were acclimatized to the animal house environment prior to the experiments and were given pelleted rat chow and water *ad libitum* except in fasting experiment where rats were given only water *ad libitum*. The rats were cycled in a 12 hour light (L, 0700-1900) and 12 hour dark (D, 1900-0700) period for two weeks and were routinely killed at L3, except in the starvation experiment where the rats were killed at L9. The rats were killed by decapitation in all experiments except in *in vivo* experiments, where the rats were anesthetized and livers were removed.
EXPERIMENTAL DESIGN

All the experimental groups contained five rats each. The daily dose of all the substances given through gastric intubation was divided into two halves, one half was given in the morning and the other half in the evening, unless stated otherwise.

2.2.1. *Tocotrienol Rich Fraction (TRF) Treatment to Normolipidemic Rats*: The experimental groups were given TRF in 1 ml normal saline at the dose stated below.

**Normolipidemic, treated groups:**
- Given 100 mg TRF/rat/day for one week.

**Normolipidemic, control groups:**
- These rats were given 1 ml normal saline for one week.

2.2.2. *TRF Treatment to Hyperlipidemic Rats*: These rats were given atherogenic diet (AD) containing 5% saturated fat, 1% cholesterol and 0.5% cholic acid, administered through gastric intubation for the induction of hyperlipidemia. The specified doses of TRF/rat/day were given in 1 ml normal saline.

**Hyperlipidemic, treated groups:**
- Given AD for three weeks alongwith 100 mg TRF/rat/day.
- Given AD for three weeks and 25 mg TRF for 5 days after its withdrawal.
- Given AD for three weeks and 50 mg TRF for 5 days after its withdrawal.
- Given AD for three weeks and 25 mg TRF for 8 days after its withdrawal.
- Given AD for three weeks and 25 mg TRF for 8 days after its withdrawal.
- Given AD for three weeks and 12 mg TRF for 8 days after its withdrawal.

**Hyperlipidemic, control groups:**
- Given AD for three weeks alongwith 1 ml normal saline.
(ii) Given AD for three weeks and 1 ml normal saline for 5 days after its withdrawal.

(iii) Given AD for three weeks and 1 ml normal saline for 8 days after its withdrawal.

2.2.3. **Dietary Stress Experiments**: Following dietary manipulations were done:

**Treated groups**:

(i) Lovastatin Treated group; given 7.5 mg lovastatin solution in 2 ml normal saline/rat/day for one week.

(ii) Cholestyramine Treated group; given 300 mg cholestyramine suspension in 2 ml normal saline/rat/day for one week.

(iii) Lovastatin and Cholestyramine Treated group; given 7.5 mg lovastatin and 300 mg cholestyramine in saline, for one week.

**Control group**:

Given 2 ml normal saline/rat/day for one week.

2.2.4. **Fasting Experiment**: Rats were starved for the following duration:

- **Fasted rat groups**:
  
  (i) 21 hours.
  
  (ii) 36 hours.

- **Control group**:
  
  Given free access to diet and water.

2.2.5. **Mevalonolactone (MVL) Treatment Experiment**: 100 mg MVL solution in 1 ml normal saline/rat was given in a single oral dose.

- **Treated groups**:
  
  (i) Given MVL 30 minutes before killing.
  
  (ii) Given MVL 4 hours before killing.

- **Control group**:
  
  Given 1 ml normal saline, 30 minutes before killing.
EXPERIMENTAL PROCEDURES

2.3.1. Isolation and Purification of Tocotrienol Rich Fraction (TRF) from Refined Rice Bran Oil (RBO)

TRF was extracted by stirring RBO in methanol for 1 h. The minimum amount of methanol essential for extraction of maximum amount of TRF had been worked out to be 7 ml per gram of RBO. Methanol layer containing TRF was evaporated at 65°C under vacuum. Purified TRF was isolated from crude TRF as reported (He *et al.*, 1996) with minor modification. 100 g silica gel (230-400 mesh) suspended in hexane was layered on a 500 ml sintered glass funnel connected to a water aspirator tube for generating a mild vacuum. The silica gel was washed with 500 ml of hexane and then loaded with 0.5 g of crude TRF in 20 ml of hexane. The gel was washed with 1.5 l of hexane in order to remove non-tocotrienol/tocopherol components such as triacylglycerides, sterols, etc. Purified mixtures of tocotrienol (T₃) and tocopherol (T) was then rapidly eluted with diethyl ether. The elution was speeded by the application of vacuum produced by water aspirator. The solvent was evaporated under vacuum in order to obtain highly purified mixture of T₃ and T. The silica gel was regenerated by thoroughly washing with methanol, which removes all the bound components and reused again for the purification of crude TRF.

2.3.2. Preparation of Cytosol

Male albino rats were decapitated, liver was taken out and placed in chilled saline. The tissue was washed in cold saline, weighed and homogenized in a warring blender (30 seconds) in two volumes of buffer A (20 mM Tris-HCl pH 7.0, 80 mM KCl, 200 mM sucrose, 0.1 mM TPCK, 0.1 mM TLCK, 0.1 mM benzamidine and 0.02 mM leupeptin). The homogenate was centrifuged at 15000 x g for 20 min at 4°C. The pellet was discarded and the post-mitochondrial supernatant was centrifuged at 100,000 x g for 1 h at 4°C. The cytosol was obtained as supernatant
and the microsomal pellet was used for the preparation of washed microsomes. The cytosol from liver were aliquoted and stored frozen at -20°C for future use. In order to separate the two molecular forms, of CEH, hepatic cytosol was first fractionated 0-40% with ammonium sulfate. The pellet was dissolved in buffer A, stored frozen and used for high molecular weight form (69 kDa) CEH assay. The post 40% supernatant was further fractionated to a final ammonium sulfate concentration of 80%. The 40-80% ammonium sulfate pellet obtained after centrifugation was dissolved in buffer A and used for the assay of low molecular weight form (45kDa) CEH activity. Same procedure was used for the preparation of pancreas and brain cytosol, which were used in immunological studies.

2.3.3. Preparation of Microsomes

The microsomal pellet was suspended in ten volumes of buffer B (40 mM potassium phosphate, pH 7.2, 100 mM Sucrose, 50 mM KCl and 30 mM potassium EDTA) by vortexing, as described by Edwards and Gould (1972). The suspension was centrifuged at 100,000 x g for 1 hr at 4°C. The washed microsomal pellet was frozen at -20°C for further use.

2.3.4. Solubilization of HMG-CoA Reductase

Solubilization was performed as described by Edwards et al. (1979). The frozen microsomes were allowed to thaw at 37°C. An equal volume of 50% glycerol in buffer C (buffer B with 10 mM DTT) was added which was preheated to 37°C. The suspension was homogenized with 10 downward passes of a hand driven all glass Potter-Elvehjem homogenizer and then incubated at 37°C for 1 h. The suspension was diluted 3 fold with the same buffer preheated to 37°C to a final glycerol concentration of 8.3%, rehomogenized with 10 downward passes of the homogenizer pestle, and centrifuged at 100,000 x g for 60 min at 25°C. The supernatant which contained solubilized reductase was decanted and used for enzyme assay or stored at -20°C for further use.
2.3.5. **Preparation of Substrates of CEH**

PNP-acetate, PNP-butyrate, PNP-caprylate, PNP-palmitate, PNP-stearate or CO (cold or radiolabelled) was dissolved in acetone at the concentration of 4 mM (40X) and was used as droplet dispersion. Emulsion of CO was prepared as previously reported by Noel et al. (1983). 10 µmole of the substrate was dissolved in 25 µl of benzene to which 175 µl of Triton-X100 was added and the contents were vortexed. 40 µl of this emulsion was added dropwise by a hamilton syringe to 1 ml of a rapidly vortexing saline.

2.3.6. **Assay of CEH**

An improved and sensitive colorimetric method for the assay of CEH was developed in our laboratory which utilizes p-nitrophenyl ester of palmitic acid as a substrate. CO and other p-nitrophenyl esters of short and long chain fatty acid were also used in comparative studies.

Hydrolysis of acetone solutions of PNP-acetate, PNP-butyrate, PNP-caprylate, PNP-palmitate and PNP-stearate by CEH enzyme was measured colorimetrically in 100 mM tris-HCl, pH 7.0, containing 50-400 µg protein of cytosol or ammonium sulfate fractions as a source of enzyme and 0.1 mM PNP-acetate, PNP-butyrate, PNP-caprylate, PNP-palmitate or PNP-stearate in a total volume of 250 µl. The reaction was started by the addition of substrate and incubated for 10 min at 37°C. The reaction was stopped by incubating the assay tubes in an ice chilled water bath. The tubes were centrifuged at 10,000 rpm for 2 min at 4°C and the absorbance of the hydrolytic product, p-nitrophenol in the supernatant was measured at 405 nm in a semi-autoanalyser (Vitalab 31, The Netherlands). The CEH activity was calculated by utilizing p-nitrophenol as a standard. One unit of enzyme activity was defined as the amount of CEH required to hydrolyze one nmole of substrate into p-nitrophenol and corresponding fatty acids per min. at 37°C.
When the above method of CEH assay was compared with other previously published radiometric or colorimetric methods, incubation conditions described for PNP-esters (above) were used. Hydrolysis of radiolabeled CO (0.1 mM; 60,000 dpm) was measured by the radiometric method as described previously (Chen et al., 1986). When CO substrate (0.1 mM) was used as emulsion or acetone solution, free cholesterol released, following incubation with cytosolic CEH, was extracted in isopropanol and measured colorimetrically involving an enzyme coupled assay as described previously (Gallo, 1981, Noel et al., 1983). One unit of CEH activity is defined as the activity which hydrolysed one n mole of cold or radiolabeled CO per min at 37°C.

2.3.7. Assay of HMG CoA Reductase

HMG-CoA reductase activity in solubilized fraction of microsomes was assayed spectrophotometrically by the method of Kleinsek et al. (1977) with minor modifications. The activity of the solubilized HMG-CoA reductase was determined at 37 °C in a total volume of 0.25 ml using Beckman DU 640 spectrophotometer. Assay was carried out in buffer C containing 0.16 M potassium phosphate, pH 6.8, 200 mM KCl, 4 mM EDTA and 20 mM 2-Mercaptoethanol. The reaction cell contained 100-200 µg protein, 0.2 mM NADPH and 0.1 mM HMG-CoA, whereas, control cell contained only 100-200 µg protein and 0.2 mM NADPH. Before the addition of HMG-CoA, the tubes were preincubated for 15 minutes. The reaction was stopped in cold water bath at 4°C after incubation for 5 min at 37°C. The change in absorbance from zero time was monitored at 340 nm against blank containing all the reagents except NADPH. The linear rate of oxidation of NADPH in control cell was subtracted from the linear rate obtained in the reaction cell.

One unit of enzyme activity is defined as the amount of enzyme required to oxidize 2 nmoles of NADPH or 1 nmole of mevalonate per minute at 37 °C.
2.3.8. Purification of CEH

The cytosol was first fractionated by ammonium sulfate precipitation into 0-40% ASF and 40-80% ASF in order to separate high and low molecular forms of CEH, respectively. The two fractions were dissolved in buffer A and aliquots were run separately on the non-denaturing gel electrophoresis at 4°C. At the end of the run, the gels were cut into 1 mm slices and a portion of each slice was assayed for the presence of CEH activity. The gel slices from several runs, containing peak activities of high and low molecular weight forms of CEH were pooled separately and the enzyme protein was eluted by incubating the gel slices in 20 mM ammonium bicarbonate for 16 h at 4°C. The pooled enzyme fractions of the two molecular forms of CEH were concentrated in 30,000 molecular weight cut Centricon concentrators by centrifugation at 7000 x g at 4°C.

2.3.9. Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed as described by Laemmli (1970). However, in certain runs, the stacking gel was omitted. The staining was routinely done by commassie brilliant blue R250 (0.05%) solution in water containing 25% isopropanol and 10% acetic acid. The destaining was done with a solution of 10% acetic acid and 10% methanol in water. For detecting very small amount of protein on gels, Comassie blue-G colloidal concentrate was used.

2.3.10. Non-Denaturing (non-SDS) PAGE

Non SDS-PAGE was done like SDS-PAGE except SDS and 2-mercapto-ethanol were omitted.

2.3.11. Enzyme-Linked Immunosorbant Assay (ELISA) for CEH Mass

ELISA for CEH mass was developed in our laboratory. Assays were done in 96 well maxisorp E plates (Nalge NUNC). Wells were filled with 3-9 µg protein of 0-40% ASF or 10-50 µg protein of 40-80% ASF, diluted in 0.05 M carbonate-bicarbonate buffer pH 9.6, to a volume of 300 µl. This was allowed to sit overnight
at 4°C. The protein was then removed and the wells were washed one time each with Tween-Tris buffered saline (TTBS; 20 mM Tris-HCl, 500 mM NaCl, pH 7.4, 0.05% Tween 20) and Tris buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.4). The wells were then filled with 300 μl non fat dry milk 5% in TBS. This was allowed to incubate for 3-4 h at room temperature. The blocking solution was then removed and plates were washed one time each with TTBS and TBS, respectively. 100 μl of primary antibody, anti-rat hepatic CEH antiserum at 1:200 dilution for 0-40% ASF containing 69 kDa CEH and anti-rat pancreatic CEH IgG at dilution of 1:10,000 for 40-80% ASF containing 45 kDa CEH, was added to corresponding wells and were incubated for 1-2 h at 37°C. Wells were again washed once with TTBS and once with TBS, after the removal of primary antibody. After washing, 100 μl of secondary antibody conjugate, goat anti rabbit IgG-HRP conjugate, at 1:2000 dilution was added and left for 1-2 h at room temperature. Again washing was done once with TTBS and TBS, respectively. Colour was developed by the addition of 3,3',5,5'-tetramethyl benzidine-peroxidase system and incubation at 37°C for 30 min. The colour development was terminated by the addition of 100 μl of 1 M phosphoric acid. The absorbance was read at 450 nm by automatic microplate reader (MR-600 Dynatech USA).

The standard plot for the estimation of cytosolic high (69 kDa) and low (45 kDa) molecular forms of CEH proteins was made by using several concentrations (0.2-5.0 ng) of purified high and low molecular forms of CEH or commercial rabbit liver and bovine pancreatic CE hydrolases. The primary antibodies used were anti-rat hepatic CEH antiserum and anti-rat pancreatic CEH IgG, respectively.

2.3.12. Western Blotting

Western blotting was performed as described in Millipore transfer membrane user guide. SDS-PAGE of 20-25 μg protein of cytosol, 0-40% or 40-80% ASF or 1-2 μg of purified proteins was performed. After electrophoresis,
the gels washed with double distilled water and were electroblotted 3 h, at 4°C at 200 volts, onto Immobilon-Polyvinylidene difluoride (PVDF) membranes using the BioRad transblot cell. The transfer buffer contained 25 mM Tris, 192 mM glycine, pH 8.3, 20% v/v methanol and 0.02% SDS. After the transfer was over, the PVDF membrane was taken out and kept in 5% non fat dry milk dissolved in TBS (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 2 h at room temperature. The membrane was washed with TTBS (10mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.02% Tween 20) and TBS. The primary antibody, diluted in TBS was added and incubated at room temperature for 2 h with constant shaking. After removing the primary antibody, membrane was washed thrice with TTBS and once with TBS (8 min each). Secondary antibody HRP conjugate diluted in TBS was added after washing and allowed to stay for 1 h at room temperature. The membrane was washed again thrice with TTBS and once with TBS (8 min each). The membrane was then submerged in HRP colour substrate system (50 mM Tris-HCl, pH 8.0, 0.48 mM 4-chloro-1-naphthol, 200 mM NaCl and 17% methanol). The reaction was started with the addition of H₂O₂ to the concentration of 0.01% v/v. The colour was allowed to develop until the bands became visible. The substrate was removed and the membrane was dried and photographed the same day.

2.3.13. Estimation of Total, Free and Esterified Cholesterol in Liver Homogenate

The total lipid was extracted from the liver homogenates as described by Folch et al. (1957). Total and free cholesterol were determined by the method of Annino and Giese (1976). 1 ml of the rat liver lipid extract was added to 4 ml isopropanol and mixed by vortexing. 1 ml of this mixture was taken and treated with ferric chloride reagent (70 mg ferric chloride hexahydrate per 100 ml of glacial acetic acid), mixed immediately followed by the addition of 2 ml of sulfuric acid with instant mixing. Parallel controls (no lipid extract) and cholesterol standards were also run. The absorbance was taken at 550 nm, 5 min after the addition of sulfuric acid.
For free cholesterol, 1 ml of the lipid extract-isopropanol mixture was treated with 2 ml of acetone and one ml of 1% digitonin (1 g digitonin in 100 ml of 60:40 ethanol water mixture). The tubes were allowed to stand for 30 min in ice bath and then centrifuged at 4000 rpm for 10 min. The supernatant was completely decanted. the precipitate which contained free cholesterol was again washed with 3 ml of acetone and dissolved in 1 ml isopropanol, after centrifugation. The separated free cholesterol was then determined by same procedure as used for total cholesterol.

Esterified cholesterol, for the corresponding tissue, was estimated by subtracting the value of free cholesterol from total cholesterol. The absorbance reading were taken on Beckman DU 640 Spectrophotometer.

2.3.14. Protein Estimation

The protein was determined by the method of Bradford (1976), using bovine serum albumin as standard. Crude samples such as homogenates, cytosol and ammonium sulfate fractions of cytosol were first precipitated with 20% trichloro acetic acid. The pellets were dissolved in 1N NaOH and suitable aliquots were used for protein determination.

2.3.15. Neuraminidase Treatment

High (69 kDa) and low (45 kDa) forms of purified CEH protein fractions (approximately 1.5 μg protein) were incubated with 0.1 unit of neuraminidase for 18 h at 37°C. The protein samples were then electrophoresed on SDS-gel with the corresponding untreated CEH protein and molecular weight standards.

2.3.16. Statistical Analysis

Statistical analysis of data was done by employing two tailed Student t-test (Bennet and Franklin, 1967), P values less than 0.05 were considered significant.