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SHORT COMMUNICATIONS

High density lipoprotein is a scavenger of superoxide anions

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The structural damage to organic compounds of a wide variety may occur as a consequence of oxidative stress inflected by reactive oxygen species [1]. The detoxication of these free radicals occurs by non-enzymatic as well as enzymatic antioxidants [2, 3] so as to inhibit lipid peroxidation in tissues. Blood high density lipoprotein (HDL) is known to play vital roles in the transport and regulation of lipids for delivery to cell and tissues [4]. This lipoprotein has an anti-atherogenic role and is involved with the mechanism of reverse transport and removal of cholesterol from atrial smooth muscle cells [5, 6]. Along with distribution and transport of cholesterol, HDL is known to prevent the cytotoxic action of hyperlipemic β-lipoproteins [7].

Our recent observations indicated that HDL protects the lipid peroxidation of hyperlipemic β-lipoproteins *in vitro* and the antioxidant effect of HDL was comparable to that of butylated hydroxy toluene, a known chemical antioxidant [8]. The purpose of the present study was to demonstrate the free radical scavenging property of HDL against the generation of O_2^- anions in both enzymic and non-enzymic systems *in vitro*. The effect of HDL as a protector of free radical species was compared with that of BHA. The present communication also shows how, and in what way, the anti-oxidant action of HDL differs from that of butylated hydroxyanisole (BHA).

Materials and Methods

Xanthine oxidase (EC 1.1.3.22), peroxidase ex horseradish (EC 1.11.1.7), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine diphosphonucleotide reduced (NADPH), cacodylic acid, heparin and dextran sulphate M_r 500,000 were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and other chemicals used were of analytical grade.

Blood was withdrawn from normal healthy donors. The serum was fractionated into very low density lipoprotein (VLDL) low density lipoprotein (LDL) and HDL by polyanionic precipitations [9]. HDL fraction was further purified [10]. Protein was estimated by standard procedure [11].

Boiled rat liver microsomes (1.8 mg protein) and 250 μM sodium ascorbate (to stimulate the lipid peroxidation) were added with known concentrations of HDL or BHA in 0.1 M phosphate buffer pH 7.4. Simultaneously same amounts of microsomes, HDL or antioxidant were incubated separately and mixed together, similar to that of experimental tubes, to serve as respective controls. Malonaldehyde (MDA) contents in both sets were measured by the thiobarbituric acid method [12].

Superoxide anions were generated by 0.4 mM pyrogallol in 2.5 mL air equilibrated 50 mM Tris cacodylic acid buffer pH 8.2 containing 1 mM diethyltriamine pentaacetic acid [13]. The rate of auto-oxidation of pyrogallol, as shown by an increase in extinction/min at 420 nm was compared with the reaction mixture containing different concentrations of test substances (HDL or BHA). Another system employed for the generation of O_2^- anions comprised of 10 μM phenazine methosulphate, 60 μM NADH and 40 μM NBT in 0.1 M pyrophosphate buffer pH 9.2 to a final volume of 2.5 mL [14]. After 90 sec incubation in the presence of HDL or antioxidant, the reaction was terminated by adding 1.0 mL CH_3COOH and the colour was read at 560 nm, against respective blanks.

The effect of test substances on the generation of O_2^- anions in two different enzymic systems, xanthine–xanthine oxidase [15] and resorcinol–peroxidase [16] was investigated. Xanthine oxidase activity, in the solution containing 80 μM of xanthine in 0.1 M phosphate buffer pH 7.4, 0.03 units/mL of xanthine oxidase added with known concentrations of test substances to a final volume of 2.5 mL, was assayed spectrophotometrically at 295 nm. The change in extinction/min was compared with the reaction mixture which did not include HDL or BHA. The influence of test samples on NBT reduction by O_2^- anions was seen in the reaction mixture containing 0.03 units/mL of xanthine oxidase, 80 μM of xanthine and 40 μM NBT in 0.1 M phosphate buffer pH 7.4. The reaction was stopped by adding glacial CH_3COOH and extinction was read at 560 nm against respective blanks. Similarly a system comprised of 120 μM NADPH, 20 μM resorcinol, 12 μM $MnCl_2$ in 0.05 M Tris–HCl buffer pH 7.4 and known concentrations of HDL or BHA was added with 1.0 unit/mL of peroxidase. The rate of oxidation of NADPH was measured at 340 nm and compared with references without test substances.

Results and Discussion

Non-enzymic lipid peroxidation in microsomes. The influence of HDL (15–300 pmol/mL) on ascorbate induced lipid peroxidation in rat liver microsomes was investigated (Table 1). HDL when added in concentrations ranging from 15 pmol to 167 pmol was found to protect in a concentration dependent manner against MDA formation. However, further addition of HDL up to 300 pmol did not cause any more decrease in the generation of MDA. In order to compare the antioxidant potential of HDL, effect of various concentrations (0.1–1.0 μmol/mL) of BHA were also studied. It was found that this antioxidant at the concentrations of 0.35 μmol and 0.7 μmol provided 50 and 100% protection respectively ($P < 0.01$) against the lipid peroxidation of microsomes. Flavonoids and superoxide dismutase are also known to inhibit the ascorbate induced lipid peroxidation in rat liver microsomes [17].

It has been reported that ascorbate alone, in absence of traces of Fe^{2+} chelated by EDTA is unable to catalyse the lipid peroxidation suggesting that fatty acid hydroperoxides undergo metal ion catalysed decomposition to fatty acid-free radicals which may degrade to malondialdehyde [18, 19].

Non-enzymic generation of superoxide anions. The effect of HDL on non-enzymic generation of O_2^- anions by (i) peroxidation of pyrogallol and (ii) NADH–phenazine methosulphate–NBT system, were tested at various concentrations from 15–300 pmol/mL in the reaction mixture (Table 2). The peroxidation of pyrogallol was shown to be inhibited by HDL in a concentration dependent manner which was maximum at 105 pmol. Similarly phenazine methosulphate–NADH generated O_2^- anions which were measured by their action on NBT. Addition of HDL at various concentrations significantly inhibited the reduction of NBT and maximum effect was observed by 105 pmol.

Table 1. The influence of HDL on malonaldehyde generation in rat liver microsomes stimulated with ascorbate

Final concentration of HDL (pmol/mL)	Reference (nM MDA/mg protein)	Experimental (nM MDA/mg protein)	% Inhibition by HDL
15	1.17 ± 0.04	1.08 ± 0.01	7.7
30	1.57 ± 0.02	1.33 ± 0.04	15.3
45	1.93 ± 0.03	1.46 ± 0.05	24.4
60	2.35 ± 0.07	1.50 ± 0.07	36.2
75	2.66 ± 0.07	1.37 ± 0.12	48.5
112	3.42 ± 0.12	1.62 ± 0.07	52.6
150	4.05 ± 0.06	1.25 ± 0.04	69.0
166	4.69 ± 0.11	1.10 ± 0.02	76.6

Assays were performed as described in Materials and Methods.
Values are mean ± SD of six separate observations. $P < 0.01$.

Table 2. Effect of HDL on non-enzymic superoxide anions generating systems

System studied	Concentration of HDL (pmol/mL)	% Inhibition by HDL
Generation of O_2^- anions by auto-oxidation of pyrogallol in the presence of cacodylic acid [13]	105	61
Generation of O_2^- anions by phenazine metho sulphate–NADH as assayed by reduction of NBT [14]	105	44

Assays were performed as described in Materials and Methods.
Values are mean of six separate observations. $P < 0.01$.

Table 3. Effect of HDL on enzymic superoxide anions generating systems

System studied	Concentration of HDL (pmol/mL)	% Inhibition by HDL
Generation of O_2^- anions during oxidation of xanthine by xanthine oxidase [15]	60	43
Reduction of NBT by O_2^- anions generated by xanthine-oxidation [15, 14]	90	54
Generation of O_2^- anions by peroxidase–resorcinol–$MnCl_2$ system as assayed by the oxidation of NADPH [16]	120	50

Assays were performed as described in Materials and Methods.
Values are mean of six separate observations. $P < 0.01$.

The final product of pyrogallol auto-oxidation, as measured by the increase in optical density of the reaction mixture, is purpurogallin which has been inhibited by the addition of SOD but not by catalase showing H_2O_2 is not involved in the auto-oxidation mechanism [20]. Various concentrations (0.1–2.0 μmol/mL) of BHA showed no effect against the generation of O_2^- anions in both the non enzymic systems.

Enzymic generation of superoxide anions. The inhibitory action of HDL on the enzymic generation of O_2^- anions are shown in Table 3. The xanthine–xanthine oxidase system generated O_2^- was measured by the reduction of NBT. The enzymic oxidation of xanthine to uric acid was shown to be inhibited by HDL at all concentrations from 15 to 300 pmol/mL in the reaction mixture and maximum protection was observed at 60 pmol. Similarly, HDL also inhibited the reduction of NBT in the above system and the maximum effect was observed at 90 pmol. However, BHA at the concentrations ranged from 0.1 to 2.0 μmol/mL did not inhibit the formation of uric acid or reduction of NBT. The scavenging property of HDL for NBT reduction in the xanthine–xanthine oxidase system may be due to the dismutation of O_2^- anions. Recently it has been reported that the implications for the antioxidant activity of propranolol may be due to its inhibitory action on xanthine oxidase [21].

The reaction mixture, comprised of peroxidase, resorcinol and $MnCl_2$, generated O_2^- anions which oxidized NADPH into NADP. HDL (15–300 pmol/mL) when added in the above reaction mixture for the oxidation of NADPH caused significant inhibition, which was maximum at 120 pmol. The inhibitory action of HDL in above systems was compared to that of BHA at various concentrations from 0.1 to 2.0 μmol/mL. It was observed that this compound at 0.15 μmol caused 50% inhibition against the oxidation of NADPH ($P < 0.01$). The prevention of oxidation of NADPH to NADP by both HDL and BHA may be due

to their inhibitory effect on peroxidase activity or their binding with Mn^{2+}. HDL a natural biochemical antioxidant reported for the first time would be of great importance like α-tocopherol and ascorbic acid to interrupt the radical chain reactions of lipid peroxidation in body. The mechanism of action of HDL may also be similar to that of some plasma proteins namely transferrin, lactoferrin, caeruloplasmin and albumin which kept Cu^{2+} or Fe^{2+} ions safely sequestered to prevent them from participating in the free radical reactions [22].

In summary, present work describes a new property of HDL to act as a scavenger of O_2^- free radicals *in vitro*. This lipoprotein prevents both enzymic and non-enzymic generation of O_2^- anions as evidenced by inhibition of xanthine oxidase, peroxidase, peroxidation of pyrogallol and phenazine methosulphate–NADH reaction. Ascorbate stimulated MDA formation in microsomes has been shown to be suppressed by HDL and these effects are comparable with that of BHA.

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